

5-13-2011

Novel Systems for the Functional Characterization of Genes Related to Paclitaxel Metabolism in Taxus Cell Cultures

Khamkeo Vongpaseuth

University of Massachusetts Amherst, kvongpaseuth@cns.umass.edu

Follow this and additional works at: https://scholarworks.umass.edu/open_access_dissertations



Part of the [Plant Biology Commons](#)

Recommended Citation

Vongpaseuth, Khamkeo, "Novel Systems for the Functional Characterization of Genes Related to Paclitaxel Metabolism in Taxus Cell Cultures" (2011). *Open Access Dissertations*. 408.

https://scholarworks.umass.edu/open_access_dissertations/408

This Open Access Dissertation is brought to you for free and open access by ScholarWorks@UMass Amherst. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of ScholarWorks@UMass Amherst. For more information, please contact scholarworks@library.umass.edu.

**NOVEL SYSTEMS FOR THE FUNCTIONAL CHARACTERIZATION OF
GENES RELATED TO PACLITAXEL METABOLISM IN *TAXUS* CELL
CULTURES**

A Dissertation Presented

by

KHAMKEO VONGPASEUTH

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Plant Biology Graduate Program

© Copyright by Khamkeo Vongpaseuth 2011

All Rights Reserved

**NOVEL SYSTEMS FOR THE FUNCTIONAL CHARACTERIZATION OF
GENES RELATED TO PACLITAXEL METABOLISM IN *TAXUS* CELL
CULTURES**

A Dissertation Presented

by

KHAMKEO VONGPASEUTH

Approved as to style and content by:

Susan C. Roberts, Chair

Elsbeth L. Walker, Member

Magdalena Bezanilla, Member

Elsbeth L. Walker, Director, Plant Biology
Graduate Program

DEDICATION

"What makes a man who he is? Is it the worst things he's ever done? Or the best things he wants to be? When you find yourself lost in life and you're nowhere near where you were going, how do you find a way from the person you've become to the one you know you could have been?"

This dissertation is dedicated to my mother.
She never stopped believing that I'd find my way.

ACKNOWLEDGMENTS

It is a deeply held conviction that worthwhile accomplishments are rarely undertaken alone. Accordingly, there are many people who deserve recognition here.

Firstly, I want to thank Susan C. Roberts and Elsbeth L. Walker for mentoring me. From them, I learned how to conduct myself both in the laboratory and in my professional life. I am also thankful to Jennifer Normanly for her mentoring on all things biochemistry. I would also like to recognize Magdalena Bezanilla for our many conversations regarding science. Additionally, I want to acknowledge past and present members of the Roberts and Walker research groups. Their support never faltered and was always appreciated.

Secondly, I want to thank those crazy chemical engineers that were my housemates at 89 North Prospect Street. Living in Amherst would have been lonely if not for the friendship of Karl Hammond, Jared Hjersted, Christos Karanikas, and Bernard Panzarella. I also want to thank Nancy Ann Price for always holding my hand. Our shared memories are my most cherished from western Massachusetts.

A special thanks goes out to the Brattleboro ice hockey group for showing me rink life beyond college hockey.

Lastly, I am indebted to those people that have been with me since the beginning. My family (Boukhay, Somchai, Thongbay, and Tongsay); childhood friends (Michael Vergara and Carl Kozub); and friends from TCNJ (Jon Santos and Renee Bednarcik) — they all carried me at some point during my life. For that, I am forever grateful.

ABSTRACT

NOVEL SYSTEMS FOR THE FUNCTIONAL CHARACTERIZATION OF GENES RELATED TO PACLITAXEL METABOLISM IN *TAXUS* CELL CULTURES

MAY 2011

KHAMKEO VONGPASEUTH, B.S., THE COLLEGE OF NEW JERSEY

M.Sc., UNIVERSITY COLLEGE DUBLIN, IRELAND

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Susan C. Roberts

Human society has benefited greatly from plant secondary metabolites, often utilizing a variety of compounds as dyes, food additives, and drugs. In particular, pharmaceutical development has benefited greatly from plant secondary metabolites. One example of this utility is paclitaxel, a highly substituted diterpene approved in the treatment of breast cancer, ovarian cancer, non-small cell lung cancer, and the AIDS-related Kaposi's sarcoma. Demand of paclitaxel is likely to increase, due to the current examination of paclitaxel in numerous clinical trials against a variety of other cancers.

Taxus cell culture represents a production source of paclitaxel to meet future demand. However, paclitaxel production through *Taxus* cell culture is often variable and low. Targeted metabolic engineering of *Taxus* to produce superior paclitaxel-accumulating lines is a viable strategy to address variable and low yields. To facilitate the production of genetically engineered *Taxus* cell lines, stable transformation is required to examine the long-term effect of gene expression *in vitro*. Additionally, suitable transient transformation systems are necessary to characterize novel *Taxus* genes related to paclitaxel accumulation.

A transient particle bombardment-mediated transformation protocol was developed to introduce transgenes into *Taxus* cells *in vitro*. Additionally, agroinfiltration in *Nicotiana benthamiana* was examined as a system to express genes related to paclitaxel biosynthesis and lead to the accumulation of the first dedicated taxane, taxa-4(5), 11(12)-diene. In regard to stable transformation, an *Agrobacterium*-mediated transformation protocol was developed, though this method requires further optimization for reliability and increased transformation efficiency. These transformation technologies will aid in the creation of elite paclitaxel-accumulating *Taxus* cell lines.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	v
ABSTRACT	vi
LIST OF TABLES	xii
LIST OF FIGURES.....	xiii
 CHAPTER	
1. INTRODUCTION AND BACKGROUND.....	1
1.1 Plant Secondary Metabolites	1
1.1.1 Paclitaxel Discovery and Application	2
1.1.2 <i>Taxus</i> Cell Culture.....	3
1.1.3 Limitations of <i>Taxus</i> Cell Culture	3
1.2 Traditional Methods of Increasing Paclitaxel Accumulation in Culture	5
1.2.1 Callus and Suspension Culture.....	5
1.2.2 Elicitation to Increase Paclitaxel Yields in Culture.....	6
1.3 Non-traditional Methods for Increasing Paclitaxel Yields in Culture	7
1.3.1 Elucidation of the Paclitaxel Biosynthetic Pathway.....	7
1.4 Gene Transfer in Plants	8
1.5 Research Goals	9
 2. MATERIALS AND METHODS	 18
2.1 Materials.....	18
2.2 Cell Culture	18
2.2.1 <i>Taxus</i> Cell Line Maintenance.....	18
2.2.2 Transfer of <i>Taxus</i> Cells from Suspension to Solid Media.....	19
2.2.3 Fresh Weight Measurements for <i>Taxus</i> Cells on Solid Media.....	20
2.2.4 Elicitation.....	20
2.3. Biochemical Analyses.....	20
2.3.1 <i>Taxus</i> Viability	20
2.3.2 Taxane Sample Preparation and Analysis Through HPLC.....	21
2.3.3 Taxane Sample Preparation with SPE and Analysis Through GC-MS	22
2.3.4 LUC and GUS assays	24
2.4 Molecular Techniques.....	25
2.4.1 RNA Purification.....	25
2.4.2 First Strand cDNA Synthesis	25

2.4.3 Binary Plant Expression Vectors.....	25
2.5 Transformation Methodology.....	27
2.5.1 <i>Agrobacterium</i> -mediated Transformation	27
2.5.2 Particle Bombardment	31
2.5.3 Agroinfiltration in <i>N. benthamiana</i>	32
2.6 Statistical Analyses	34
3. DEVELOPMENT OF A PARTICLE BOMBARDMENT-MEDIATED TRANSFORMATION SYSTEM FOR <i>TAXUS</i> CELLS IN CULTURE.....	36
3.1 Introduction	36
3.2 Background.....	36
3.2.1 Benefits of a Transient Transformation System.....	36
3.2.2 Particle Bombardment-mediated Transformation.....	37
3.3 Materials and Methods.....	38
3.4 Results and Discussion.....	38
3.4.1 Choice of Reporter	38
3.4.2 Effect of Rupture Disc Pressure and Stopping-Screen-to- Target Distance.....	39
3.4.3 Effect of DNA and Gold Microcarrier Concentration.....	40
3.4.4 Effect of Multiple Bombardments.....	41
3.4.5 Effect of Microcarrier Diameter.....	42
3.4.6 Expression of Different Reporters and Promoters	43
3.5 Conclusions	44
4. AGROINFILTRATION IN <i>NICOTIANA BENTHAMIANA</i> AS A SYSTEM FOR THE CHARACTERIZATION OF PACLITAXEL BIOSYNTHETIC GENES.....	53
4.1 Introduction	53
4.1.1 Paclitaxel Biosynthesis	53
4.1.2 Established Methods used to Characterize Taxane Biosynthetic Genes	57
4.1.3 Transient Transformation in Plants as a Protein Characterization System	59
4.1.4 Agroinfiltration in <i>Nicotiana benthamiana</i>	61
4.1.5 Agroinfiltration in <i>N. benthamiana</i> for Taxane Accumulation.....	63
4.2 Materials and Methods.....	64
4.3 Results	64
4.3.1 Agroinfiltration as a Method to Deliver Transgenes into <i>N.</i> <i>benthamiana</i> Tissue	64
4.3.2 Nonadecane Recovery from a Flow-Through Solid Phase Extraction Column.....	65
4.3.3 Taxa-4(5), 11(12)-diene Accumulation in <i>N. benthamiana</i> Following Agroinfiltration with Taxadiene Synthase	66

4.3.4	Co-agroinfiltration of TASY and T5 α H Leads to the Absence of Taxa-4(5), 11(12)-diene and Taxa-4(20),11(12)-dien-5 α -ol	67
4.3.5	Determination that Co-agroinfiltration is not Failing to Co-transform the Same Cells in <i>N. benthamiana</i>	68
4.3.6	Determination that T5 α H is Utilizing Taxa-4(5), 11(12)-diene as a Substrate Following Co-agroinfiltration of TASY and T5 α H	68
4.4	Discussion and Conclusions	71
4.4.1	Accumulation of Taxa-4(5), 11(12)-diene Following Agroinfiltration with TASY in <i>N. benthamiana</i>	71
4.4.2	Conversion of Taxa-4(5), 11(12)-diene into a Novel Product by T5 α H Following Co-agroinfiltration of TASY and T5 α H.....	72
5.	DEVELOPMENT OF AN <i>AGROBACTERIUM</i> -MEDIATED TRANSFORMATION PROTOCOL FOR <i>TAXUS</i> CELLS IN CULTURE.....	87
5.1	Introduction	87
5.1.1	Variability in <i>Taxus</i> Cell Culture	87
5.1.2	<i>Agrobacterium</i> -mediated Transformation	87
5.1.3	Targeted Metabolic Engineering of <i>Taxus</i>	89
5.2	Materials and Methods.....	90
5.3	Results	90
5.3.1	Examination of the Effect of Cefotaxime in <i>Taxus</i> Cells in Culture.....	90
5.3.2	Examination of Hygromycin B Selection in <i>Taxus</i> Cells in Culture.....	91
5.3.3	Examination of Glufosinate Ammonium Selection in <i>Taxus</i> Cells in Culture.....	92
5.3.4	Examination of Traditional <i>Agrobacterium</i> -mediated Transformation	93
5.3.5	Examination of a Novel Method of <i>Agrobacterium</i> -mediated Transformation	95
5.3.6	Dry Co-Cultivation of <i>Agrobacterium</i> and <i>Taxus</i> Cells in Culture.....	96
5.4	Discussion and Conclusions	97
5.4.1	Selection of <i>Taxus</i> Transformants.....	97
5.4.2	Prospectives for the Generation of Transgenic <i>Taxus</i> Cell Lines.....	100
6.	CONCLUSIONS AND RECOMMENDATIONS.....	120
6.1	Introduction	120
6.2	Transient Introduction of Transgenes Through Particle Bombardment	120

6.2 Agroinfiltration of <i>Nicotiana benthamiana</i> with Taxadiene Synthase and Taxadiene-5 α -hydroxylase	122
6.3 <i>Agrobacterium</i> -mediated Transformation of <i>Taxus</i>	124
6.4 Prospects of Metabolic Engineering in <i>Taxus</i> Cell Culture	126
BIBLIOGRAPHY	129

LIST OF TABLES

Table

2.1. Primer pair sequence.....	35
3.1. Effect of plasmid DNA and gold microcarrier concentrations on transient luciferase activity in P991 and CO93D cell cultures.....	49
5.1. Media composition and protocol for of Gelvin <i>Agrobacterium</i> virulence gene priming.....	111
5.2. Combination of factors related to the traditional liquid-liquid co-cultivation of <i>Taxus</i> spp. cell culture and <i>Agrobacterium tumefaciens</i>	112
5.3. Combination of factors related to reproduction of the <i>Agrobacterium</i> -mediated transformation protocol presented in Ketchum et al. (2007).....	116
5.4. Combination of factors related to the traditional liquid-liquid co-cultivation of <i>Taxus</i> spp. cell culture and <i>Agrobacterium tumefaciens</i> following the priming of <i>Agrobacterium</i> virulence genes as described in Gelvin (2005).....	117
5.5. Combination of factors related to the dry co-cultivation of <i>Taxus</i> spp. cell culture and <i>Agrobacterium tumefaciens</i> following the priming of <i>Agrobacterium</i> virulence genes as described in Gelvin (2005).....	119

LIST OF FIGURES

Figure

1.1.	Structure of paclitaxel.....	12
1.2.	Needles and arils of <i>Taxus brevifolia</i>	13
1.3.	Simplified paclitaxel biosynthetic pathway.....	14
1.4.	<i>Taxus</i> suspension cultures.....	15
1.5.	First half of the paclitaxel biosynthetic pathway.....	16
1.6.	Second half of the paclitaxel biosynthetic pathway.....	17
3.1.	Native <i>Taxus</i> green autofluorescence.....	46
3.2.	Native <i>Taxus</i> GUS activity.....	47
3.3.	Effect of rupture disc pressure and target distance on transient transformation of four <i>Taxus</i> cell lines with either a 35S:luciferase construct or PZP221 empty vector construct.....	48
3.4.	Effect of multiple bombardments on the transient transformation of <i>Taxus</i> cell lines P991 and CO93D with either a 35S:luciferase construct or PZP221 empty vector construct after three bombardments per sample.....	50
3.5.	Effect of microcarrier diameter on transient transformation of <i>Taxus</i> cell lines P991 and CO93D with either a 35S:luciferase construct or PZP221 empty vector construct after three bombardments per sample.....	51
3.6.	Transient transformation of two <i>Taxus</i> cell lines with a UB:dsRed construct.....	52
4.1.	GUS activity in <i>Nicotiana benthamiana</i> following agroinfiltration.....	75
4.2.	Demonstration of multiple transformation events following co-agroinfiltration with three different fluorescent reporter constructs.....	76
4.3.	Total ion count and selected ion monitoring of nonadecane (10pg/μl) after purification through an SPE silica column and GC-MS analysis.....	77

4.4.	GC-MS comparison of <i>Nicotiana benthamiana</i> leaf tissue from non-SPE treated samples and SPE treated samples.....	78
4.5.	GC-MS analysis of taxa-4(5), 11(12)-diene following extraction of <i>Nicotiana benthamiana</i> agroinfiltrated with 35S:TASY.....	79
4.6.	GC-MS analysis of <i>Nicotiana benthamiana</i> sample agroinfiltrated with 35S:T5αH.....	80
4.7.	GC-MS analysis of <i>Nicotiana benthamiana</i> sample co- agroinfiltrated with 35S:TASY and 35S:T5αH.....	81
4.8.	GC-MS analysis of <i>Nicotiana benthamiana</i> sample co-agroinfiltrated With TASY and the mCherry fluorescent protein(1:1).....	82
4.9.	5(12)-oxa-3(11)-cyclotaxane.....	83
4.10.	GC-MS analysis of <i>Nicotiana benthamiana</i> sample co-agroinfiltrated with TASY and T5αH (9:1).....	84
4.11.	GC-MS analysis of <i>Nicotiana benthamiana</i> sample co-agroinfiltrated with TASY and the mCherry fluorescent protein.....	85
4.12.	Quantification of taxa-4(5), 11(12)-diene following co-agroinfiltration of <i>Nicotiana benthamiana</i> with either 35S:TASY and 35S:mCherry or 35S:TASY and 35S:T5αH.....	86
5.1.	<i>Taxus</i> cell line viability on 0, 150, or 300 µg/ml of cefotaxime following a 28-day incubation on solid Gamborg B5 media.....	104
5.2.	Molecular structure of hygromycin B and glufosinate.....	105
5.3.	<i>Taxus</i> cell line viability on various concentrations of hygromycin B following a 28-day incubation on solid Gamborg B5 media.....	106
5.4.	<i>Taxus</i> cell line viability on various concentrations of glufosinate following a 28-day incubation on solid Gamborg B5 media.....	107
5.5.	Standard liquid/liquid co-cultivation between <i>Taxus</i> and <i>Agrobacterium tumefaciens</i>	108
5.6.	Priming of <i>Agrobacterium tumefaciens</i> prior to <i>Taxus</i> co-cultivation.....	109
5.7.	GUS activity in <i>Taxus</i> cell line PO93X following dry co-cultivation.....	110

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Plant Secondary Metabolites

Secondary plant metabolic products are an evolutionary adaptation to a sessile life habit and permit a plant to further interact with its environment. These natural products, however, also play a role in human society, and are often utilized as dyes, food additives, and drugs. The interwoven relationship between plant secondary metabolic products and humans likely precedes recorded history, though this intimacy has been documented through the millennia in texts ranging from Mesopotamian cuneiform clay tablets (circa 2600 BC) to the present day *Dictionary of Natural Products* (Ji et al., 2009). Plant secondary metabolic products that have commercially endured through time include caffeine, codeine, digitoxin, morphine, and acetylsalicylic acid (Balunas and Kinghorn, 2005).

Plant secondary metabolites continue to play vital roles in human society, particularly in the development of pharmaceuticals. For example, over 70% of new chemical structures are identified as originating from higher plants (Gibson, 1995). Due to the lack of any commercialized product resulting from industrial application of combinatorial chemistry (Ortholand and Ganesan, 2004), the emphasis has again shifted to plant products as muses for targeted drug design and novel therapeutics (Vongpaseuth and Roberts, 2007).

1.1.1 Paclitaxel Discovery and Application

A massive bio-screening program initiated by the National Cancer Institute (NCI) in 1958 examined more than 110,000 plant-derived compounds for anti-cancer activity. One result of this ambitious survey is paclitaxel, a highly substituted diterpene originally isolated from the bark of *Taxus brevifolia* (Figure 1.1 and Figure 1.2, respectively). Paclitaxel is unique among the antimitotic agents such as colchicine and the *Vinca* alkaloids; paclitaxel prevents depolymerization of the microtubule network essential to cellular division, inducing apoptosis in affected cells (Oberlies and Kroll, 2004). Due to this exclusive mechanism of action, the Food and Drug Administration (FDA) approved paclitaxel, either alone or in conjunction with other chemotherapeutic agents, in the treatment of breast cancer, ovarian cancer, non-small cell lung cancer, and the AIDS-related Kaposi's sarcoma (Exposito et al., 2009a). In addition to FDA-approved treatments involving paclitaxel, numerous clinical trials are pending examining the effects of paclitaxel against a variety of cancers (<http://www.cancer.gov>).

Despite receiving considerable attention for its ability to stabilize microtubules, paclitaxel has also been shown to target and inhibit other subcellular structures, such as Bcl-2, a human protein that prevents a cell from undergoing apoptosis and DNA polymerase α (Malonga et al., 2005; Oshige et al., 2004). Several studies have utilized paclitaxel in the treatment of medical issues not related to cancer, such as Alzheimer's disease and preventive narrowing of arteries after balloon angioplasty (Doraiswamy and Xiong, 2006; Nawarskas and Osborn, 2005).

1.1.2 *Taxus* Cell Culture

Given the growing pharmaceutical applications of paclitaxel and second-generation paclitaxel-derived compounds, sales of paclitaxel are expected to exceed three billion US dollars worldwide in the next few years (Croteau, 2006). Currently, the majority of paclitaxel is supplied through a semi-synthetic process utilizing 10-deacetyl baccatin III (10-DAB), a naturally occurring taxane extracted from the needles of various yew species, as a substrate (Zhong, 2002). Although the semi-synthetic route represents a renewable supply source, the manufacturing process involves 11 chemical transformations along with 13 solvents and therefore poses significant environmental issues. Additionally, there is significant variability in 10-DAB yield amongst yew species, also limiting the efficacy of the semi-synthetic process (van Rozendaal et al., 2000; Yang et al., 2008; Zhao et al., 2006).

Taxus cell suspension culture offers the potential to supply increased quantities of paclitaxel compared to whole tissue extraction or semi-synthesis, while avoiding the use of harmful solvents (Kolewe et al., 2008). Compared to total synthesis, *Taxus* cell suspension culture is ideal for scale-up to the commercial level (Kolewe et al., 2010). *Taxus* cell suspension culture already represents a success of plant cell culture technology in the U.S. with its proven feasibility by Bristol-Myers Squibb and Phyton Biotech, Inc., a DFP Pharmaceuticals company, in supplying paclitaxel for Bristol-Myers Squibb's TAXOL® formulation.

1.1.3 Limitations of *Taxus* Cell Culture

Taxus cell culture is not without its limitations. The *Taxus* system, like numerous plant cell culture systems, displays variability and limited accumulation of desirable

secondary metabolites (Dornenburg and Knorr, 1995; Exposito et al., 2009a; Frense, 2007; Gibson et al., 1993; Kolewe et al., 2008; Roberts, 2007; Tabata, 2004; Tabata, 2006). The reason for this variation is not currently known, though it is speculated that genetic or epigenetic causes may be responsible (Xiang et al., 2008). While recent evidence in the regulation of paclitaxel biosynthesis in *Taxus* cell culture suggests a putative transcriptional control mechanism (Nims et al., 2007), a lack of complete information regarding paclitaxel global metabolic processes, such as transport and degradation, and a lack of complete genome sequence information for *Taxus* limits full exploitation in cell culture. The majority of what is known about paclitaxel metabolism centers on paclitaxel biosynthesis (Figure 1.3). Understanding global paclitaxel metabolism is required to optimize accumulation in *Taxus* cell culture. A complete understanding of paclitaxel metabolism, including the molecular controls of enzymatic reactions leading to paclitaxel biosynthesis, subcellular transport of paclitaxel and its precursors, and *in vivo* degradation is necessary before superior paclitaxel accumulation in cell culture can be fully realized (Vongpaseuth and Roberts, 2007).

Two general strategies have been applied to address issues of paclitaxel variability and yield in culture. Traditional strategies generally include selection of superior *Taxus* cell lines for paclitaxel accumulation, and precursor feeding or elicitor challenging in cell culture. Non-traditional methods involve the utilization of technological advances in molecular biology and biochemistry to clone, identify, and over-express/silence potential genes of interest in paclitaxel biosynthesis.

1.2 Traditional Methods of Increasing Paclitaxel Accumulation in Culture

1.2.1 Callus and Suspension Culture

Plant calli are undifferentiated proliferating masses of somatic cells growing on solid media. Studies on plantlet regeneration, organogenesis, and culture of *Taxus* fungal anticulture variants have typically resulted in lower paclitaxel yields than *Taxus* cell culture, and will not be reviewed here (Datta et al., 2006; Jaziri et al., 1996; Miller et al., 2008; Zhang et al., 2008). Calli may be induced from various explant sources (e.g., needles, bark, and embryos), with cell lines previously initiated from *T. baccata*, *T. brevifolia*, *T. chinensis*, *T. cuspidata*, *T. canadensis*, *T. wallichiana*, *T. x media*, and *T. yunnanensis* (Furmanowa et al., 2000; Gibson et al., 1993; Jaziri et al., 1996; Pyo, 2004; Vongpaseuth and Roberts, 2007). To further optimize cell growth and to facilitate media extraction of secreted taxanes, *Taxus* calli are often transferred from solid to liquid media and incubated with continuous agitation, creating *Taxus* cell suspension cultures (Figure 1.4). Suspension cultures are preferred to callus cultures for commercialization because they are more amendable to scaling (Navia-Osorio et al., 2002; Zhong, 2002).

Optimization of media composition for *Taxus* cell suspension culture has been thoroughly studied and reviewed (Gibson et al., 1993; Gibson, 1995; Tabata, 2006; Zhong, 2002). Among the different plant growth media used, increased nitrogen content, high sucrose content (2-3%), and inclusion of the plant growth hormones auxin and cytokinin are generally beneficial for growth and paclitaxel production. In addition, paclitaxel accumulation is significantly increased when *Taxus* suspension cultures are incubated in darkness (Fett-Neto, 1995).

1.2.2 Elicitation to Increase Paclitaxel Yields in Culture

An elicitor is any compound, biotic or abiotic in origin, that causes an increase in plant secondary product accumulation through a transcriptional upregulation of genes related to the defense response. Abiotic elicitors utilized in the *Taxus* system include the rare earth metal lanthanum, silver nitrate, or vanadyl sulfate (Furmanowa et al., 2000; Luo and He, 2004; Wu et al., 2001). Biotic elicitors can be grouped into two distinct classes. In the first class, the elicitor is an uncharacterized mixture of compounds that are often derived from the fungal cell wall extract of non-pathogenic fungi, such as *Aspergillus niger* or *S. cerevisiae* (Ciddi et al., 1995; Khosroushahi et al., 2006; Linden and Phisalaphong, 2000; Luo and He, 2004; Qin and Lan, 2004; Wang, 2001; Yuan, 2002). In the second class, the elicitor is a purified compound. This may represent isolated components of the fungal cell wall, such as chitin, chitosan, or N-acetylchitohexaose (Linden and Phisalaphong, 2000; Zhang et al., 2000). Proteins such as cellulase have also been used to increase paclitaxel production in *Taxus* suspension cultures, though this is likely due to increasing paclitaxel release from the cell wall (Roberts et al., 2003). Lipids or lipid derivatives have also played a role in increasing paclitaxel yield from *Taxus* suspension cultures. Though arachidonic acid and salicylic acid have been used with some success, jasmonic acid and its methyl ester, methyl jasmonate, have been shown to increase paclitaxel accumulation significantly in suspension culture (Baebler et al., 2002; Ciddi et al., 1995; Ketchum and Gibson, 1996; Wang et al., 2007; Yukimune et al., 1996). In addition, synthetic jasmonates with novel functional group substitutions are having success in increasing paclitaxel yield in *Taxus* cell cultures (Qian et al., 2004; Qian et al., 2005). In all cases, however, growth of *Taxus*

in culture after elicitation is reduced compared to unelicited controls, indicating a transition from primary metabolism to secondary metabolism preceding increased taxane accumulation (Exposito et al., 2009b; Kim et al., 2005; Kim, 2004).

1.3 Non-traditional Methods for Increasing Paclitaxel Yields in Culture

1.3.1 Elucidation of the Paclitaxel Biosynthetic Pathway

Paclitaxel is a cyclic and poly-oxygenated diterpene containing several acyl group substitutions, an oxetane ring, and a phenylisoserine C13 ester side chain that differentiates it not only in structure from other taxanes, but also anti-mitotic activity.

Paclitaxel biosynthesis begins with the generation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), isoprene precursors originating from the plastidial methyl-erythritol phosphate (MEP) pathway. Metabolic inhibitor studies utilizing the cytoplasmic isoprene inhibitor lovastatin and the plastidial isoprene inhibitor fosmidomycin, in addition to radioactive labeling/incorporation experiments with glucose, have shown conclusively that the taxane ring system of paclitaxel is not of mevalonate origin (Eisenreich et al., 1996; Palazon et al., 2003). This is not surprising, considering that many plant terpenoids are synthesized via the MEP pathway (Lichtenthaler, 1999).

Dedicated paclitaxel biosynthesis is a complex metabolic pathway that can be regarded in three distinct stages. The first stage comprises formation of the first committed paclitaxel precursor, taxadiene, and oxygenation of the C5 position of the core taxane ring system (Figure 1.5). The second stage involves functional decoration of the core taxane ring and epoxide formation between the C4 and C5 double bond (Figure 1.5).

The third stage involves acylation steps on route to advanced taxane and paclitaxel biosynthesis, along with side chain generation, conjugation, and modification (Figure 1.6). The majority of paclitaxel-related genes cloned from *Taxus* were identified using degenerate primer-based PCR, differential display, and cDNA library production (Ketchum, 2006; Vongpaseuth and Roberts, 2007). Detailed description of the paclitaxel biosynthetic pathway has previously been published (Croteau, 2006; Ketchum, 2006; Vongpaseuth and Roberts, 2007) and will not be discussed here. However, further discussion of the pathway can be found in the introduction of Chapter 4.

1.4 Gene Transfer in Plants

Methods for gene transfer in plant systems include utilization of *Agrobacterium*, polyethylene glycol (PEG), electroporation, and biolistics. While each of these approaches has been used with success in plant systems, *Agrobacterium* and biolistics have been utilized with the most success in gymnosperm species such as *Taxus* (Klein et al., 1992; Nehra et al., 2005; Rakoczy-Trojanowska, 2002). Each method possesses advantages and disadvantages that specifically qualify it for stable or transient transformation.

The plant pathogen, *Agrobacterium tumefaciens*, is the vector used in *Agrobacterium*-mediated transformation of aerial plant tissues. Evolution has conferred *Agrobacterium* with native machinery, the *vir* proteins, that transfers and integrates a portion of its genome (T-DNA) into a plant host genome (Gelvin, 2003a). Manipulation of these *vir* genes, such as induction by the bacterial hormone acetosyringone, is central to the success of *Agrobacterium*-mediated transformation of plants (Gelvin, 2006; Lacroix et al., 2006; Tzfira and Citovsky, 2006; van der Fits et al., 2000). While

development of an *Agrobacterium*-mediated transformation protocol in *Taxus* would allow for the generation of stable transformants, transformation efficiencies are often not high enough to permit rapid regeneration of plant material to allow for functional characterization of novel genes and assays for their respective metabolic products.

Biolistics, or particle bombardment-mediated transformation, is another system routinely used to transform gymnosperms. Whereas *Agrobacterium*-mediated transformation relies on the concerted effort of several *Agrobacterium vir* proteins to transfer DNA into a plant genome, biolistics achieves this same end with DNA-coated microcarriers and extreme velocity. Relative to other plant transformation systems, biolistics has been utilized to transform a broader range of plant species (Lorence and Verpoorte, 2004). In addition, one major advantage of biolistics is the capacity to precipitate several plasmids onto an individual microcarrier, enabling the delivery of multiple genes into a single cell (Nelson et al., 2007; Sawers et al., 2006). However, biolistic does not regularly result in integration of exogenous DNA into the plant genome, and when integration does occur, it often is complex with multiple and inverted insertion events (Klein et al., 1992; Rakoczy-Trojanowska, 2002). A relatively high transformation efficiency and complex insertions qualifies biolistics for transient assays, where detection of gene products is neither long term in duration nor dependent on stable transformation.

1.5 Research Goals

The motivation for this research is the facilitation of increased paclitaxel accumulation in *Taxus* cell culture. Molecular approaches have yielded a plethora of information regarding novel genes associated with paclitaxel biosynthesis, such as

CYP450s and transcriptional regulators, and transcriptional bottlenecks in metabolic flux on route to paclitaxel accumulation in culture. However, to fully exploit this increase in knowledge, both stable and transient transformation strategies must be developed and implemented in *Taxus*, so that fluctuations in paclitaxel accumulation variability and yield can be addressed through the directed metabolic engineering of superior paclitaxel-accumulating *Taxus* lines. Considering the variability in paclitaxel accumulation in cell culture, the directed metabolic engineering of *Taxus* is a promising avenue to supply paclitaxel for increasing future demand, both for chemotherapeutic purposes and novel treatments utilizing this potent mitotic inhibitor.

The research presented here is directed towards both the development of a protocol for the stable transformation of *Taxus*, and the development of transient transformation systems to characterize novel *Taxus* genes associated with increased paclitaxel accumulation in culture. These research accomplishments towards these goals are:

- (1) Developed an optimized biolistic protocol for the delivery of transgenes into *Taxus*, which could be used to transiently transform *Taxus* to characterize a novel transcription factor associated with paclitaxel accumulation (Chapter 3).

- (2) Developed an agroinfiltration system in *Nicotiana benthamiana* for the enzymatic characterization of novel *Taxus* CYP450s (Chapter 4).

- (3) Determined antibiotic susceptibility in *Taxus*, and developed an *Agrobacterium*-mediated transformation protocol for *Taxus* (Chapter 5).

Chapter 6 (Conclusion and Recommendations) considers the future direction of work in this research area. While significant progress was made with regards to the work

discussed in this dissertation, there are additional obstacles to overcome before the potential of the *Taxus* cell culture system can be realized.

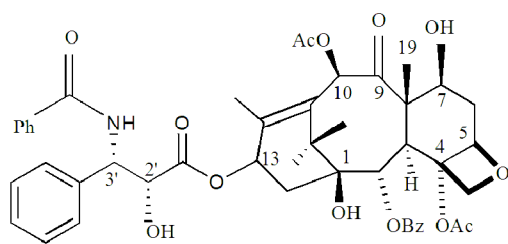


Figure 1.1: Structure of paclitaxel



Figure 1.2: Needles and arils of *Taxus brevifolia*

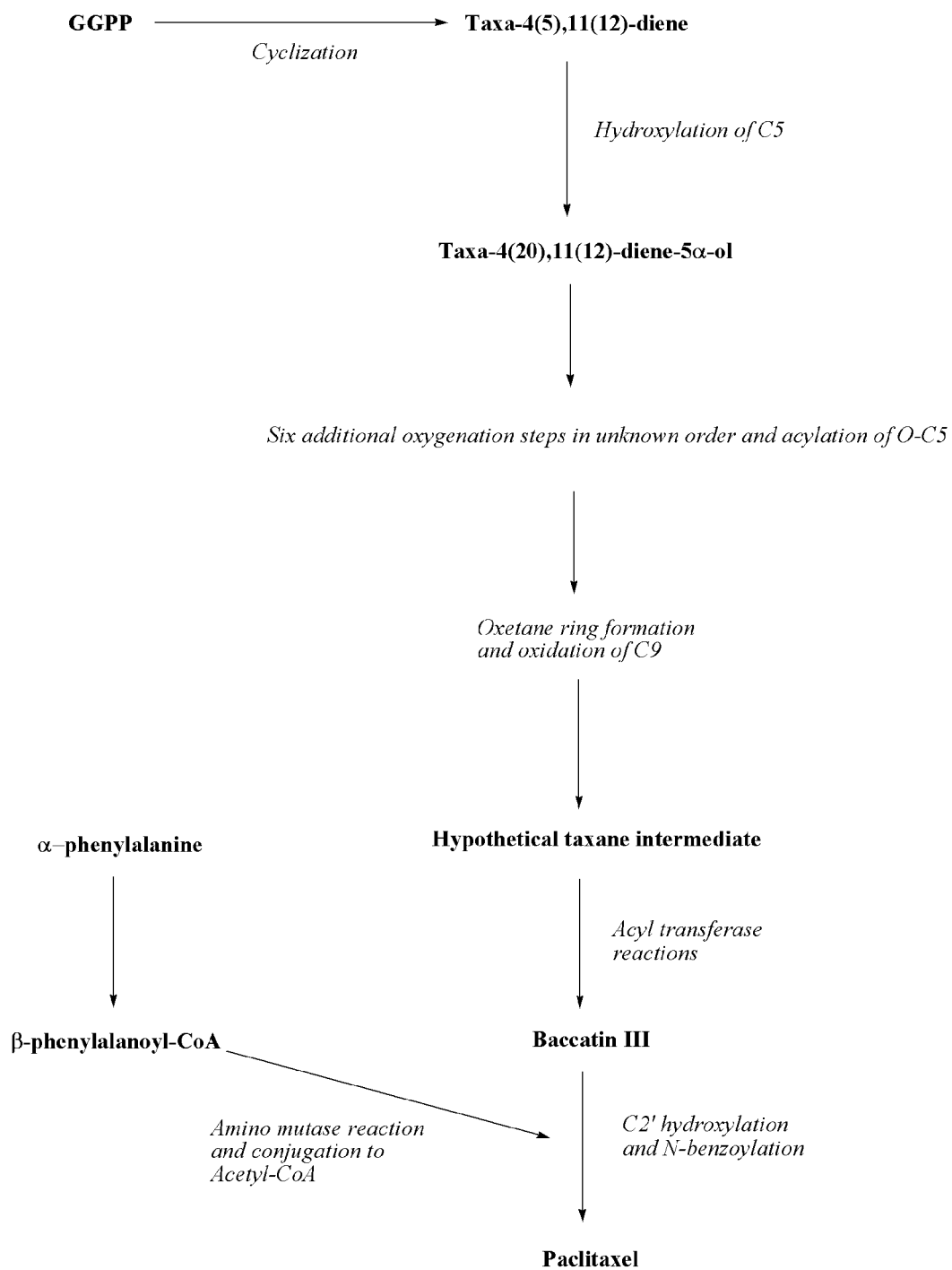


Figure 1.3: Simplified paclitaxel biosynthetic pathway



Figure 1.4: *Taxus* suspension cultures

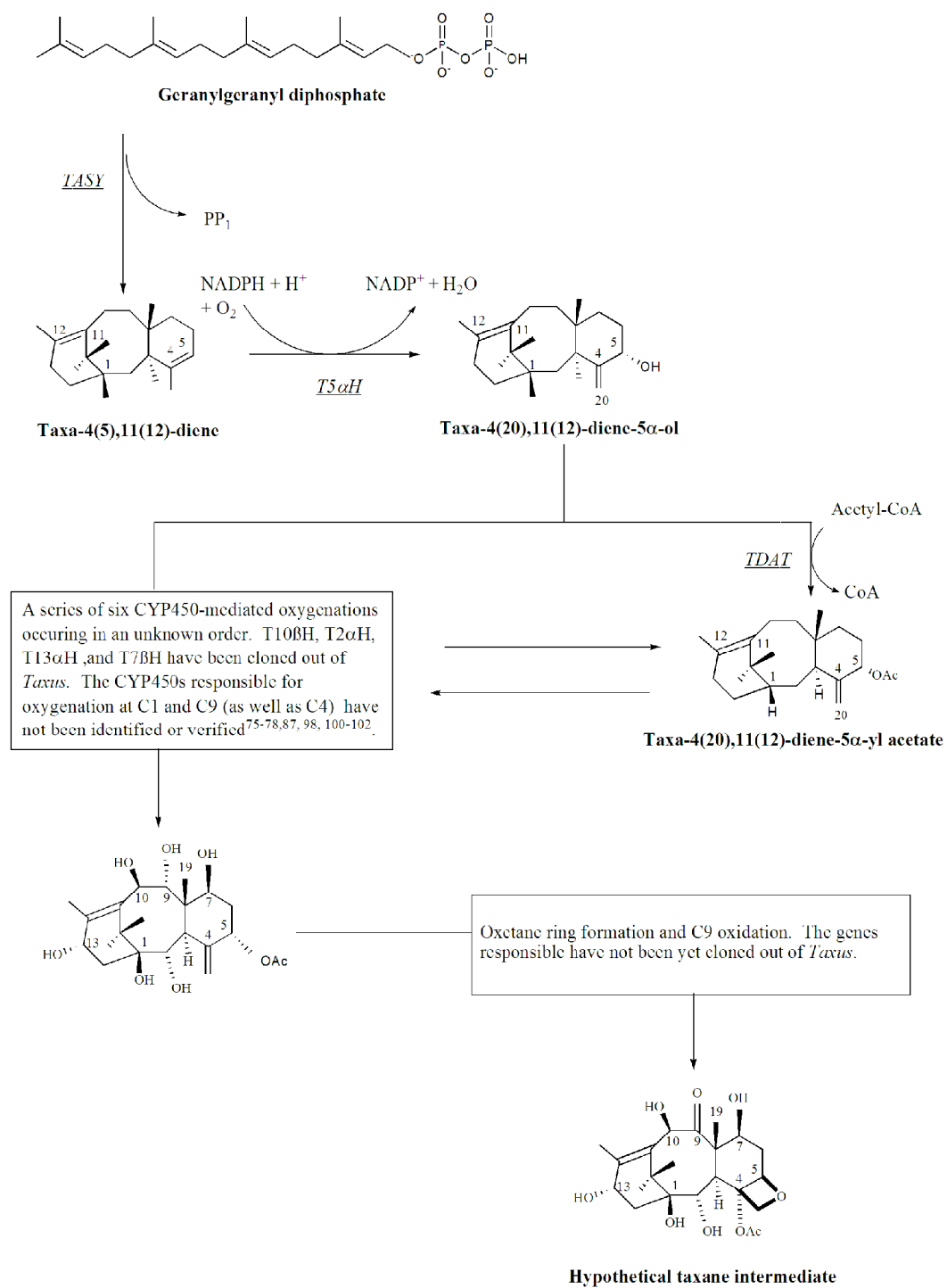


Figure 1.5: First half of the paclitaxel biosynthetic pathway

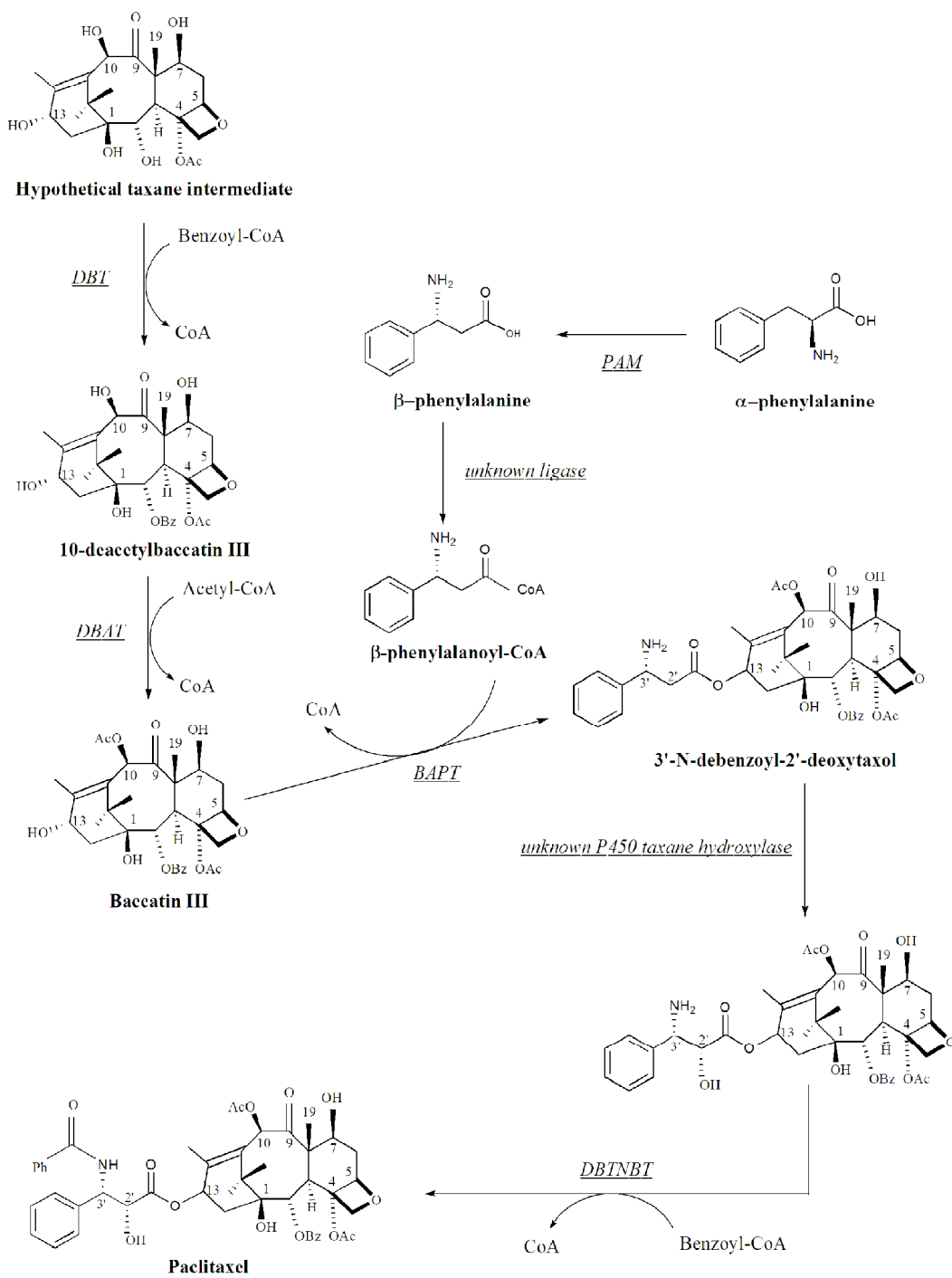


Figure 1.6: Second half of the paclitaxel biosynthetic pathway

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

10-Deacetyl baccatin III and cephalomannine were acquired from Hauser Chemical Research, Inc. (Boulder, CO), and taxane standards were prepared at a concentration of 2 mg/ml. Glufosinate ammonium was obtained from Crescent Chemical Company, Inc. (Islandia, NY). HPLC grade acetonitrile, methanol, and hexane were obtained from Fischer Scientific, Inc., and all water was supplied by a Barnstead Nanopure Infinity unit (Thermo Fisher, Inc.). All other chemicals were obtained from Sigma Chemical Co., Inc. (St. Louis, MO) unless otherwise noted. Gene products were amplified using Platinum Taq Polymerase (Invitrogen, USA) unless otherwise noted.

2.2 Cell Culture

2.2.1 *Taxus* Cell Line Maintenance

Taxus lines P991 (*T. cuspidata*), P93AF (*T. cuspidata*), PO93X (*T. cuspidata*), and CO93D (*T. canadensis*) were provided by the US Plant Soil and Nutrition Laboratory (Ithaca, NY). Suspension cultures were maintained in New Brunswick shaker/incubators (Edison, NJ) in darkness at 125 rpm and 23°C. All cultures were maintained in 125 ml glass shake flasks with Bellco (Vineland, NJ) foam closures. Every two weeks, cells were subcultured into a *Taxus* growth media, consisting of Gamborg's B5 basal salts with 20 g/l sucrose, 2.7 µM naphthalene acetic acid (NAA), and 0.1 µM benzyladenine (BA). For subculture, 10 ml of 14 day-old culture was transferred into 40 ml of fresh media with 3-4 ml of packed cell volume per flask. Glutamine (5 mM), ascorbic acid (125

mg/l), and citric acid (125 mg/l) were filter sterilized through a Millex GP 0.22 µm PES unit (Millipore, Ireland), and added post-autoclave at 5% volume of the culture volume for P991 and P93AF, and 4% volume of the culture volume for PO93X and CO93D (Vongpaseuth et al., 2007).

Most critical to the success and maintenance of *Taxus* cells, regardless of cell line, is the removal of necrotic aggregates from the cultured cells. Necrotic aggregates occur in each *Taxus* cell line and is a common occurrence in plant cell lines *in vitro*. Removal of necrotic aggregates can be undertaken at any point during the culture cycle of each *Taxus* cell line; additionally, if the abundance of necrotic aggregates increases towards the end of the two-week culture cycle, early subculture may be required to preserve *Taxus* cell line viability. Additionally, *Taxus* suspension cultures are capable of secreting pigmented stress compounds into the media, darkening the media from clear to brown/red. In this scenario, early subculture may also be necessary to preserve the integrity of the cell line, with extreme cases requiring the transfer back onto solid media to preserve the viability of the *Taxus* cell line.

2.2.2 Transfer of *Taxus* Cells from Suspension to Solid Media

Taxus cell lines P991, P93AF, PO93X, and CO93D were individually vacuum-filtered through autoclaved Calbiochem Miracloth (La Jolla, CA), and plated onto solid *Taxus* growth medium containing 14 g/l plant cell culture grade agar. A total of 0.2 g (antibiotic viability studies) or 0.75-1.0 ml packed cell volume (particle bombardment studies) was plated onto each 100 mm Petri dish approximately 0-24 hours before each experiment. In studies where antibiotic addition to the *Taxus* growth media was required, these antibiotics were added post autoclave just prior to media solidification. If not

utilized immediately, plated cells were stored at room temperature in the dark until needed for a period of time not exceeding 24 hours.

2.2.3 Fresh Weight Measurements for *Taxus* Cells on Solid Media

The fresh weight of plated cells was measured on a pre-tared 100 mm Petri dish in an Ohaus Precision Standard balance (Pinebrook, NJ). *Taxus* cells were transferred utilizing cell scrapers (Fisher Scientific, Inc.) to avoid inadvertent removal of solid media for gravimetric analysis.

2.2.4 Elicitation

The cell cultures were elicited with methyl jasmonate seven days post-transfer, unless otherwise noted (Ketchum et al., 1999). For elicitation, 40 µl of methyl jasmonate was added to 460 µl 95% (v/v) ethanol and 500 µl nanopure water. This solution was vortexed and then filtered with a Pall Acrodisc 0.2 µm PVDF filter (East Hills, NY) into a sterile 1.5 ml microfuge tube, and then aliquotted into suspension cultures to yield the desired concentration. Unelicited cell cultures were given a similar volume of ethanol/nanopure (1:1 v/v) as a negative control.

2.3. Biochemical Analyses

2.3.1 *Taxus* Viability

Cell viability was determined by staining with fluorescein diacetate (FDA) for one minute (Widholm, 1972). For viability testing, 20 µl of FDA (0.5 mg/ml stock solution in cold acetone) was added to approximately 1 ml of *Taxus* suspension culture and incubated for ten minutes at room temperature on the bench top. Analysis with

fluorescence microscopy (Zeiss LSM 510 Meta Confocal System) was used to distinguish metabolically active cells from non-metabolically active cells. Unstained cells were used as a control to determine autofluorescence, which was found to be negligible in all cases.

2.3.2 Taxane Sample Preparation and Analysis Through HPLC

High Performance Liquid Chromatography (HPLC) was used to monitor taxane concentrations in *Taxus* culture following resuspension/centrifugation sample preparation.

For sample preparation, 1000 µl of total samples (cells and media) were collected with a cut 1 ml pipette tip, and evaporated down on a Savant SpeedVac[®] Plus. The dried material was resuspended in 1000 µl acidified methanol (0.01% (v/v) glacial acetic acid in methanol). After sonication for 30 minutes, the samples were vortexed and disrupted by incubation in a sonication bath again for 30 minutes. Centrifugation for 25 minutes at 10,000 x g was followed by removal of the supernatant. The pellet was resuspended in 1000 µl of acidified methanol and subjected to a second extraction. Next, the supernatants from the two extractions were combined in a new tube and dried completely under an air stream using an Evap-o-rac (Cole Parmer, Vernon Hills, IL). The dried material was resuspended in 100 µl of acidified methanol, followed by an additional 30 minutes of sonication. Following centrifugation (25 minutes, 10,000 x g), the sample was filtered through a Pall Acrodisc 0.2 µm PVDF filter (East Hills, NY) and analyzed by HPLC.

Separation was accomplished on a Varian (Torrance, CA) Taxsil[®], 250 mm x 4.6 mm x 5 mm column equipped with guard cartridges. The mobile phase was acetonitrile

and water (50:50) at a flow rate of 1 ml/min, unless otherwise noted. The detector scanned from 210-400 nm, with peak detection at 228 nm. Paclitaxel, as with most taxanes, has a characteristic absorption peak at 228 nm.

Taxanes were identified and quantified using HPLC in combination with taxane standards provided by either Sigma Chemical Co., Inc. (St. Louis, MO) or Hauser Chemical Research, Inc (Boulder, CO). Identification of unknown peaks was based upon the comparison with known standards of retention time and UV absorption spectra.

2.3.3 Taxane Sample Preparation with SPE and Analysis Through GC-MS

Gas Chromatography-Mass Spectroscopy was utilized to monitor taxane samples from *Nicotiana benthamiana* following sample preparation through Solid Phase Extraction (SPE).

For sample preparation, four grams of *N. benthamiana* leaf tissue were ground to a fine powder in a sterilized mortar and pestle under liquid nitrogen. Ground *N. benthamiana* tissue was transferred to a 25 ml Teflon® cap-lined vial (Fisher Scientific, Inc.), followed by extraction in hexane at a concentration of 3 ml/g fresh weight over night at 4°C. Overnight extraction was performed on a tabletop shaker set to 175 RPM. Purification of the hexane extract was performed through the use of a homemade SPE flow-through column composed of a 3 ml Pasteur pipette packed with glass wool, Mallinckrodt J.T. Baker, Inc., 400 mg Silica Gel 60 (Phillipsburg, NJ), and 100 mg anhydrous magnesium sulfate. The 3 ml Pasteur pipette was packed using a small funnel crafted from weigh paper within a fume hood to avoid inhalation of both glass wool and Silica Gel 60. Following addition of each sorbent into the 3 ml Pasteur pipette, it is critical to ensure that each layer is tightly packed, so that no spaces exist between

particles or layers of sorbent. These spaces, if present, will facilitate the creation of air bubbles and column “cracking” once any solvent is in contact with the sorbents. Gentle tapping of the top of each column following sorbent addition is sufficient to ensure that no empty cavities exist between sorbent layers or particles. Do not disrupt layering between sorbents, as this will reduce efficacy of the column to bind contaminants. Preceding sample loading, the SPE flow-through column was primed with a 2 ml hexane wash. Samples were loaded onto the SPE flow-through column, and the column was rinsed with hexane until just prior to the elution of the pigmented compounds (approximately 40 ml). During flow through, it is critical to prevent column drying, as this will facilitate column cracking and the formation of air bubbles within the sorbent layers. Collected fractions were combined and evaporated under an air stream using an Evap-o-rac (Cole Parmer, Vernon Hills, IL). Samples were then resuspended in 1 ml hexane spiked with 10 pg/ μ l nonadecane prior to GC-MS injection. Samples, if not utilized immediately, were stored at -80°C in 25 ml Teflon® cap-lined vial (Fisher Scientific, Inc.)

GC-MS analyses were conducted on a JEOL MStation JMS700 High Resolution Two Sector Spectrometer with integrated GC (Tokyo, Japan). A 1 μ l hexane extract was analyzed by hot injection (270°C) on a Mallinckrodt J.T. Baker, Inc. DB-5 GC-column (30 m, 0.25 mm ID, and 0.25 μ m film thickness) (Phillipsburg, NJ). Chromatography was accomplished using He as a carrier gas at 1.0 ml/min. Samples were hot injected at 270°C. Initial run temperature in the oven was 70°C, followed by a programmed gradient to 320°C at 8°C/min, with a hold at 320°C.

Comparison to published taxane fragmentation spectra were used for identification. Taxa-4(5), 11(12)-diene was analyzed through the Selected Ion Monitoring (SIM) of ions with m/z ratios of 272, 257, 229, 123, 122, 121, and 107 (Besumbes et al., 2004). Taxa-4(20), 11(12)-dien-5 α -ol was analyzed through the SIM of ions with m/z ratios of 288, 255, 227, 185, 159, 145, and 105 (Ketchum, 2006).

2.3.4 LUC and GUS assays

Extracts for measurement of luciferase activity were prepared from *Taxus* tissue ground to a powder in liquid nitrogen, and extracted with 1.5 ml of a buffer solution containing 100 nM K₂HPO₄ and KH₂PO₄, pH 7.8, 1 mM DTT, and 20 μ g/ml leupeptin. Extraction was undertaken on ice for one minute. Samples were centrifuged 10,000 rpm at 4°C for ten minutes. Supernatant was removed and stored at -80°C until use. Luciferase activity was measured on a Turner Biosystems TD20/20 luminometer (Sunnyvale, CA) set at 70% sensitivity using luciferin as a substrate and following blanking of the instrument with an extracted *Taxus* control sample (Tao et al., 2002).

For histochemical analysis of *Taxus* or *N. benthamiana* cells bombarded or agroinfiltrated with GUS constructs, tissue was incubated in X-Gluc solution (0.0005% X-Gluc, 0.0005% dimethyl formamide, and 50 mM NaPO₄ at pH 8.6) overnight at 37°C. Histochemical verification of GUS activity was undertaken using a Leica compound light microscope.

2.4 Molecular Techniques

2.4.1 RNA Purification

Taxus cells were collected by filtration through Calbiochem Miracloth (EMD Biosciences, San Diego, CA), and stored at -80°C in 1.5 ml polypropylene tubes. RNA was extracted using the Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA), followed by DNase treatment with the DNA-free kit (Ambion, Austin, TX). Quantification of total RNA was performed with the Ribogreen fluorometric assay (Molecular Probes, Eugene, OR) and visualized on a 1X TBE agarose gel with Ethidium Bromide to confirm absence/presence of degradation..

2.4.2 First Strand cDNA Synthesis

First strand cDNA was synthesized using Superscript™ II Reverse Transcriptase (RT) (Invitrogen, USA) from 5 µg total RNA. Reaction set up was designed according to the Invitrogen recommended parameters. (PCR conditions were as follows: 95°C for 2 minutes followed by 95°C, 15 seconds; 63°C, 30 seconds, 68°C, 2.5 minutes). General instructions were followed from the Invitrogen Superscript™ II Reverse Transcriptase (RT) kit.

2.4.3 Binary Plant Expression Vectors

Numerous vectors were used in this work. Some were given/purchased, while others were constructed utilizing the Invitrogen Gateway® (GW) cloning system to generate binary plant expression vectors.

Binary plant vectors utilized in chapter 3 (Particle bombardment-mediated transformation of *Taxus* cells in culture) included 35S:LUC, UB:GUS, UB:dsRed, and

Act:GUS. 35S:LUC was provided by Dr. Elsbeth L. Walker (Plant Biology Graduate Program, UMass Amherst), and represented the firefly luciferase gene driven by the 35S (Nelson et al., 2007)(Plant Biology Graduate Program, UMass Amherst). UB:GUS and Act:GUS represent the GUS reporter gene driven by the maize ubiquitin and rice actin promoters, respectively. UB:dsRed represents the *Discosoma* species red fluorescent protein reporter gene driven by the maize ubiquitin promoter.

Binary plant vectors utilized in chapter 4 (Agroinfiltration in *Nicotiana benthamiana* as a system for the characterization of paclitaxel biosynthetic genes) included pCAMBIA1301, ER-ck (2x35S:ER-CFP), pt-yk (2x35S:plastid-YFP), pm-rk (2x35S:PM-mCherry), 35S:TASY, and 35S:T5 α H. ER-ck, pt-yk, and pm-rk were purchased from the TAIR website, and represented ER-localized cyan fluorescent protein, plastid localized yellow fluorescent protein, and plasma membrane-localized mCherry fluorescent protein, respectively (www.arabidopsis.org) (Nelson et al., 2007). pCAMBIA1301 was purchased from Cambia (Brisbane, Australia), and represented the intron-GUS reporter gene driven by the 35S promoter. Both TASY and T5 α H were amplified from methyl jasmonate-elicited *T. cuspidata* cell line P991 cDNA with primer sequences designed from GenBank nucleotide information (GenBank accession numbers U48796.1 and AY289209.2, respectively) (primer pairs shown in Table 2-1). PCR conditions utilizing Platinum Taq polymerase (Invitrogen[®]) were as follows: 95°C for 2 minutes followed by 95°C; 68°C for three minutes (for TASY) or two minutes (for T5 α H); and 72°C for 2 minutes. PCR products were TOPO-TA cloned into pCR[®]8GW/TOPO (Invitrogen[®]), and then cloned into the destination vector pH7WG2 utilizing LR Clonase II[®]. pH7WG2 was acquired from the VIB Department of Plant

Systems Biology, Ghent University (Karimi et al., 2002). TOPA-TA and LR cloning were undertaken following the methods provided by Invitrogen®.

Binary plant vectors utilized in Chapter 5 (Development of an *Agrobacterium*-mediated transformation protocol for *Taxus* cells in culture) included pCAMBIA1301, 35S:GFP, and 35S:PM-mCherry. pCAMBIA1301 was acquired as previously described. 35S:GFP represented the *Aequorea victoria* green fluorescent protein reporter gene driven by the 35S promoter, and was provided by Dr. Elsbeth L. Walker (Plant Biology Graduate Program, UMass Amherst). 35S:PM-mCherry was constructed by first amplifying the mCherry fluorescent protein reporter gene from vector pm-rk (TAIR accession number: CD3-1007) utilizing Platinum Taq polymerase (Invitrogen®) (primer pair shown in Table 2-1). PCR conditions utilizing platinum taq polymerase (Invitrogen®) were as follows: 95°C for 2 minutes; 68°C for two minutes; and 72°C for 2 minutes. PCR products were TOPO-TA cloned into pCR®8GW/TOPO (Invitrogen®), and then cloned into the destination vector pH7WG2 utilizing LR Clonase II®. pH7WG2 was acquired as previously described. TOPA-TA and LR cloning were undertaken following the methods provided by Invitrogen®.

2.5 Transformation Methodology

2.5.1 *Agrobacterium*-mediated Transformation

Agrobacterium tumefaciens strains GV3101, LBA4404, and EHA105 were maintained on Luria-Bertani (LB) (25g/L) (Fischer Scientific, Inc) solid media supplemented with 1.4% agar (and 15 µg/ml gentamycin for GV3101). *Agrobacterium* strain C56C1 was maintained in a similar fashion except for the addition of 5 µg/ml

tetracycline (in DMSO) post-autoclave to the media formulation. Unless otherwise stated, all antibiotics were dissolved in water purified by a Nanopure water purifier (Thermo Fischer, Inc.). Frozen *Agrobacterium* stocks were prepared in 15% glycerol in a sterile 1.5 microfuge tube, flash frozen with liquid nitrogen, and stored at -80°C.

Traditional *Agrobacterium* transformation involved the start of a small-scale culture. A single colony of *Agrobacterium* was picked with a sterile toothpick and incubated in LB with the appropriate antibiotic at 29°C and 250 RPM agitation for approximately 18 - 24 hours. Large scale cultures were initiated the following day, with inoculation of 100 ml LB supplemented with the appropriate antibiotic and acetosyringone (dissolved in DMSO). The large-scale *Agrobacterium* culture was grown at 29°C and 250 RPM overnight until the desired OD₆₀₀ was attained (approximately 0.8). Prior to co-cultivation with 4.5 ml of seven-day old *Taxus* suspension culture in six well plates (Fischer Scientific, USA), the *Agrobacterium* was centrifuged at 3000 RPM, and resuspended in 2 ml B5. Co-cultivation was performed in the dark at room temperature for 24 – 48 hours at 120 RPM on a New Brunswick (Edison, NJ) tabletop shaker platform. Following co-cultivation, *Taxus* cells were vacuum-washed over sterilized filter paper with 25 ml sterilized B5 four times, and plated on the modified Gamborg B5 media used to maintain the *Taxus* suspension cultures supplemented with 14 g/L agar and 300 µg/ml cefotaxime.

Agrobacterium virulence gene priming and transformation utilizing the Gelvin Protocol (Gelvin, 2006) differed from the traditional protocol previously described. First, small-scale *Agrobacterium* cultures were started in 2 ml YEP media (1% peptone; 1% yeast extract; 85 mM NaCl) supplemented with the appropriate antibiotic. These small-

scale cultures were incubated at 29°C and 250 RPM agitation for approximately 24 hours. The following day, approximately 500 µl small-scale culture was used to inoculate 50 ml of AB sucrose minimal media (0.5% sucrose; 18.69 mM NH₄Cl; 2.49 mM MgSO₄ · 7H₂O; 2.0 mM KCl; 17.22 mM K₂HPO₄; 8.34 mM NaH₂PO₄; 90 µM CaCl₂; 9 µM FeSO₄ · 7H₂O) supplemented with the appropriate antibiotic. This large-scale culture was incubated at 29°C and 250 RPM agitation until the OD₆₀₀ measured 0.8. The *Agrobacterium* was centrifuged at 3000 RPM for 10 minutes, and the pellet was resuspended in 100 ml induction media (0.5% glucose; 18.69 mM NH₄Cl; 2.49 mM MgSO₄; 2.0 mM KCl; 90 µM CaCl₂; 9 µM FeSO₄ · 7H₂O; 2 mM phosphate buffer [pH 5.6]; 50 mM MES) supplemented with 100 µg/ml acetosyringone (dissolved in DMSO). See Gelvin, 2006 for original description of the *Agrobacterium* priming protocol. This culture was incubated at room temperature in a New Brunswick tabletop shaker (Edison, NJ) for approximately 18 hours at 75 RPM in the dark. Immediately preceding co-cultivation with *Taxus* in six-well plates, the *Agrobacterium* culture was centrifuged at 3000 RPM for 10 minutes, and then resuspended in 500 µl Gamborg B5 (same as Gamborg B5 utilized to culture *Taxus* cells, except without the NAA and kinetin). Co-cultivation with 4.5 ml of seven-day old *Taxus* culture occurred at room temperature at 125 RPM in the dark for approximately 18 hours. Media replacement was performed following co-cultivation, with the removal of B5 and addition of fresh 5 ml B5 supplemented with 300 µg/ml cefotaxime. This step was repeated four times, utilizing a sterile 1 ml sterile pipette tip pressed into the corner of each well, with each replacement step followed by a 15-minute incubation at room temperature and 125 RPM. A final media replacement step was performed, followed by a two-hour incubation in the dark at

room temperature and 125 RPM. *Taxus* cells were then vacuum-washed over sterilized filter paper with 25 ml B5 supplemented with 300 µg/ml cefotaxime, and then plated onto *Taxus* growth media supplemented with 14 g/L agar and 300 µg/ml cefotaxime. These plated cells were kept at room temperature in the dark until reporter assays were undertaken, generally 2-3 days after plating.

Dry co-cultivation of *Taxus* cells and *Agrobacterium* was also undertaken. Firstly, *Agrobacterium* were primed according to the Gelvin Protocol as previously mentioned. Transfer of primed *Agrobacterium* into Gamborg B5 media must be undertaken immediately preceding co-cultivation. On the day of intended co-cultivation, 5 ml of seven-day old *Taxus* suspension culture (approximately 1 ml packed cell volume) was filtered over vacuum and autoclave-sterilized filter paper until all media was removed. The *Taxus* aggregates were then placed onto one sterilized piece of filter paper, taking care not to disrupt the vacuum-packed clump of *Taxus* aggregates. This section of sterilized filter paper containing 1 ml of *Taxus* packed cell volume was then placed into a sterile Petri dish. Once the *Taxus* cells are contained within the Petri dish, 500 µl of the primed *Agrobacterium* suspension (Gamborg B5 media) was aliquotted onto the dry *Taxus* aggregates, taking care not to disrupt the vacuum-packed clump of cells. Petri dishes were then sealed with parafilm. Dry co-cultivation occurred at room temperature in the dark for two days. Following dry co-cultivation, the still-intact clump of *Taxus* aggregates was carefully removed from the Petri dish in order for washing. Washing of the *Taxus* cells (in order to dilute away *Agrobacterium*) was then performed, with each wash consisting of 5 ml Gamborg B5 media supplemented with 300 µg/ml cefotaxime under vacuum. Care was taken during each wash to not disrupt the clump of *Taxus*

aggregates too severely. Each wash step was repeated four times. Following the final wash, the *Taxus* cells were placed onto solid Gamborg B5 media supplemented with 300 µg/ml cefotaxime. The *Taxus* cells were incubated in the dark at room temperature until the reporter assay was performed, usually two days post-plating.

2.5.2 Particle Bombardment

Prior to particle bombardment, approximately 0.5 ml of *Taxus* cells (packed cell volume) was filtered through Calbiochem Miracloth (La Jolla, CA). The cells were plated on a *Taxus* growth media supplemented with 7 g/L Agar-Agar. Within 12-24 hours, the cells were bombarded using a PDS-1000 (Bio-RAD, Hercules, CA). In addition, 1.5 ml microfuge tubes were subjected to excess charge removal by a Sigma-Aldrich Zerostat 3 anti-static gun (St. Louis, MO).

For each bombardment experiment, six milligrams of Bio-Rad 1.6 µM gold particles (Hercules, CA) was vortexed in 1 ml 70% EtOH for five minutes, allowed to settle for 15 minutes, and centrifuged for five seconds in an Eppendorf 5415. The EtOH was drawn off utilizing a 22 ¼ gauge 1 ml needle syringe (BD, USA), and the following was repeated three times: one ml sterile nanopure water was added to the pelleted microcarriers, vortexed for one minute to resuspend the pellet, allowed to settle for one minute, centrifuged for five seconds, and supernatant removed. Gold was re-suspended in 100 µl of 50% Glycerol, vortexed for five minutes and then 30µl was aliquotted into three 1.5 ml microfuge tube. In individual microfuge tubes, 4 µg of plasmid (1 µg/µl), 30 µl of freshly prepared sterile 2.5 M CaCl₂, and 12 µl of 0.1 M spermidine were added separately, each addition followed by a five second vortex. Once the final component is added, the tubes are vortexed for three minutes, allowed to settle for one minute at room

temperature, and pelleted for five seconds as before. The supernatant was drawn off utilizing a 22 $\frac{1}{4}$ gauge 1 ml needle syringe, and 84 μ l of 70% EtOH was added. This was repeated two times, followed by a final resuspension step utilizing 29 μ l of 100% EtOH. 6 μ l of this resuspended solution was aliquotted to a single Bio-Rad macrocarrier (Hercules, CA).

Taxus cultures were bombarded using a Bio-Rad PPDS-1000 (Hercules, CA) at 1100 psi, and at a stopping-screen-to-target distance of 6 cm. Each bombardment was performed on three separate samples. Additional parameters related to particle bombardment, including different stopping-screen-to-target distances, rupture disk pressures, concentration of plasmid or microcarriers, diameter of microcarrier, and frequency of bombardment/*Taxus* sample are described and examined in further detail in chapter four. Bombarded cells were allowed a two-day recovery period in the dark at room temperature before luciferase or GUS assays were performed.

2.5.3 Agroinfiltration in *N. benthamiana*

A single colony of *A. tumefaciens* strain C56C1 was picked and used to inoculate 4 ml of L-medium supplemented with 5 μ g/ml tetracycline and the appropriate antibiotic. This small-scale culture was incubated at 29°C and 250 RPM agitation for approximately 24 hours. Next, 3 ml of overnight culture was pelleted in a microfuge tube at 3000 RPM for 10 minutes. The supernatant was removed and the pellet resuspended in 3 ml MES/MgCl (10 mM MgCl, 10 mM MES pH 5.6) supplemented with 100 μ M acetosyringone. The OD₅₅₀ was measured, and the culture was diluted down to 0.2 OD₅₅₀ with MES/MgCl supplemented with 100 μ M acetosyringone. *A. tumefaciens*

strain C56C1 was allowed to incubate in the MES/MgCl supplemented with 100 μ M acetosyringone for approximately one hour in the light at room temperature on the bench top. If following this procedure with the intent of co-agroinfiltrating two different *Agrobacterium* into *N. benthamiana* tissue, then the procedure is the same for both bacterial cultures, with the exception that the combined OD₅₅₀ of the two different *Agrobacterium* cultures in the final step cannot exceed 0.2 OD₅₅₀ prior to co-agroinfiltration. Thus, any ratio of two *Agrobacterium* used in the final step is volumetric.

A sterilized razor blade was used to lightly touch the lower epidermal layer of a *N. benthamiana* leaf (approximately 7.5 cm in width) in order to create a microwound, ideally bracing the opposite upper epidermal layer with your finger. *N. benthamiana* plants were maintained in the Morrill greenhouses in top soil/vermiculite (2:1) pots and watered daily. Extreme care must be taken during this initial step to not excessively damage the *N. benthamiana* leaf; a microwound exceeding several millimeters in length is not recommended and likely to incur unneeded leaf necrosis following agroinfiltration. Additionally, care must be taken to limit penetration of the microwound into additional tissue layers of the *N. benthamiana* leaf. Penetration of the microwound only into the lower epidermal layer of the *N. benthamiana* leaf is ideal. Penetration into the mesophyll layer of the leaf is permitted if not unavoidable; however, if the razor blade cut penetrates to the upper (top) epidermal layer of the *N. benthamiana* leaf, then efficiency of the agroinfiltration will be reduced along with the encouragement of unneeded tissue necrosis following agroinfiltration. A sterile 1 ml syringe was loaded with the C56C1 MES/MgCl mixture, and was gently infiltrated into the leaf, with the syringe held tight

against the lower side of the leaf over the microwound and the upperside of the leaf braced against a finger. On average, approximately 0.1 ml of *Agrobacterium* was agroinfiltrated per microwound. Constructs contained within the agroinfiltrated *Agrobacterium* included pCAMBIA1301, ER-ck (2x35S:ER-CFP), pt-yk (2x35s:plastid-YFP), pm-rk (2x35S:PM-mCherry), 35S:TASY, and 35S:T5 α H. In regards to the age of the *N. benthamiana* leaf, older leaves are easier to infiltrate than younger leaves; however, agroinfiltration efficiency is much better when the infiltration is done on younger leaves. Agroinfiltration is more difficult in younger leaves, however, as younger leaves are more easily damaged during the creation of the microwound. Optimal leaf size is about 10 cm in diameter.

Freshly agroinfiltrated *N. benthamiana* were left in the dark at room temperature overnight. The following day, agroinfiltrated *N. benthamiana* plants were placed under fluorescent lighting following an 18- and 6-hour light/dark cycle and watered daily.

2.6 Statistical Analyses

One-way ANOVA was performed using MiniTab (MiniTab, Ltd, version 13.0). Data points are the mean of three replicates and error bars represent the standard deviation unless otherwise noted.

Table 2.1: Primer pair sequences.

TASY forward primer	ttcccctgcc tctctggagaaatggctcag
TASY reverse primer	tcatacttgaattggatcaatataaacttttcttatatagtc
T5 α H forward primer	cggcaccagggtttctgctcc
T5 α H reverse primer	catgtttgtattattcttgagcgaggattc
mCherry forward primer	aatggcaaaggatgtggaagccgttcccgg
mCherry reverse primer	cggccgctttaagatctgtacagctcgtcc

CHAPTER 3

DEVELOPMENT OF A PARTICLE BOMBARDMENT-MEDIATED TRANSFORMATION SYSTEM FOR *TAXUS* CELLS IN CULTURE

3.1 Introduction

A thorough understanding of global paclitaxel metabolism and regulation is necessary to design alternate strategies to stabilize and enhance paclitaxel accumulation in plant cell culture. Recent evidence suggests a transcriptional control mechanism (Nims et al., 2006). Transcript profiling in conjunction with metabolite analysis demonstrated potential pathway bottlenecks, since elicitation with methyl jasmonate failed to induce transcripts of key pathway genes. The genes that are not induced by methyl jasmonate are potential starting points for targeted metabolic engineering.

3.2 Background

3.2.1 Benefits of a Transient Transformation System

In order to effectively characterize unknown transcription factors associated with paclitaxel accumulation in *Taxus* spp. culture and to rapidly identify potential genes that can be used in a targeted metabolic engineering strategy for establishment of superior paclitaxel-accumulating *Taxus* spp. cell lines, a suitable transient system for the introduction of transgenes into *Taxus* spp. must be developed. With a transient system established, putative regulatory genes in taxane metabolism can be rapidly screened based on changes in taxane yield and distribution. A rapid, transient transformation system would be particularly useful in screening for potential transcription factors that induce expression of known paclitaxel biosynthetic pathway genes.

The advantages of a transient transformation system compared to stable *Agrobacterium*-mediated transformation are numerous. In addition to increased transformation efficiency, the number of genes that can be cloned into a binary vector limits *Agrobacterium*-mediated transformation, and transformation of multiple genes for gene characterization can be time consuming. However, many transient systems allow for transformation of multiple genes into a single cell. This capacity to transform multiple genes can facilitate certain aspects of gene characterization such as evaluation of the ability of putative transcription factors to induce paclitaxel biosynthetic pathway gene expression.

3.2.2 Particle Bombardment-mediated Transformation

While the advantages and capacity of *Agrobacterium* to infect a diverse group of plant species has been well documented, transformation using *Agrobacterium* remains difficult in several plant phyla, even with newly developed super-virulent and ternary *Agrobacterium* systems (Gelvin, 2003b; van der Fits et al., 2000). In particular, success with gymnosperms has been mixed. In the case of these recalcitrant conifers, such as *Picea glauca*, *P. mariana*, *P. abies*, *Pinus radiata*, *P. pinaster*, and *Larix laricina*, particle bombardment-mediated transformation has been utilized to introduce both linear and plasmid DNA constructs, with numerous reports of transient transformation (Grace et al., 2005; Kim et al., 2000; Lambardi et al., 1998; Nigro et al., 2004; Salaj et al., 2005).

Development of a reliable particle bombardment protocol requires optimization. The ability to transiently transform an existing *Taxus* cell line for characterization of genes related to paclitaxel accumulation is important, since much is not known of paclitaxel metabolism beyond biosynthesis, and factors such as transport and

transcriptional regulation remain unelucidated. Here I present a particle-mediated bombardment protocol for transient transformation of *Taxus* spp. cell cultures. I optimized bombardment parameters to reduce variability amongst replicates and demonstrated the ability of multiple promoters to drive reporter expression for β -glucuronidase (GUS), firefly luciferase (LUC), and *Discosoma* species red fluorescent protein (DsRed).

3.3 Materials and Methods

A complete description of the materials and methods is located in Chapter 2. Of particular interest is *Taxus* cell culture (2.2), biochemical analyses (2.3), binary plant expression vectors (2.4.3), and particle bombardment-mediated transformation (2.5.2).

3.4 Results and Discussion

3.4.1 Choice of Reporter

Luciferase was selected as a reporter for optimization of the transient transformation protocol due to an absence of native luciferase activity in plant systems (Luehrsen, 1992). Reporter genes such as green fluorescent protein (GFP) or β -glucuronidase (GUS) were not chosen initially due to problems with endogenous fluorescence and GUS activity. Native *Taxus* spp. cell wall autofluorescence has similar spectral characteristics as GFP (excitation at 488 nm and emission at 513 nm) and precluded GFP from being used as a real-time reporter (Figure 3.1). Native GUS activity in *Taxus* spp., which appeared only after bombardment with pPZP221, prevented similar quantification of introduced GUS constructs (Figure 3.2A). Native GUS activity in *Taxus* prevents similar quantification of introduced GUS constructs, though utilization of

an X-Gluc histochemical buffer at a pH of 8.5 can drastically reduce native GUS activity by inhibiting activity of the plant GUS enzyme (Figure 3.2B) (Gallagher, 1992).

In the studies presented here, due to the destructive nature of the luciferase assay, evaluation of stable transformation was not undertaken. Plating of putative transformants on selective media would facilitate selection of stable transformants and molecular analyses such as polymerase chain reaction (PCR) and DNA gel blotting would provide concrete evidence of stable transformation should that be desired. The goal of this research was the establishment of a transient transformation protocol and therefore experimental efforts were directed towards this goal.

3.4.2 Effect of Rupture Disc Pressure and Stopping-Screen-to-Target Distance

The effect of a 650 psi or 1100 psi rupture disc pressure on luciferase activity was first investigated utilizing 1 μ g of plasmid DNA, 60 mg/ml of 1.0 μ m gold microcarriers, and a 6 or 9 cm stopping-screen-to-target distance. Large standard deviations were observed for each cell line when comparing bombardments with the reporter construct and the empty vector control (Figure 3.3). Uneven precipitation of plasmid DNA onto the gold microcarriers has previously been documented, and was likely responsible for the large variability observed between data sets (Tee and Maziah, 2005). Only P991 bombarded at a rupture disc pressure of 1100 psi and a stopping-screen-to-target distance of 6 cm displayed significant differences in luciferase activity compared to the respective negative controls (11 ± 4.0 vs. 0.71 ± 0.76) (Figure 3.3D). In addition, P991 cellular luciferase activity at this rupture disc and stopping-screen-to-target distance combination is significantly higher than that observed with P991 cells bombarded with 35S:LUC at lower rupture disc pressure and stopping-screen-to-target combinations (Figure 3.3A,

Figure 3.3B, and Figure 3.3C). No significant differences in luciferase activity were observed for any other *Taxus* cell line bombarded with 35S:LUC at any rupture disc pressure and stopping-screen-to-target distance combination (Figure 3.3). However, CO93D displayed a trend of higher luciferase activity with increasing rupture disc pressures and stopping-screen-to-target distances (4.0 ± 3.8 vs. 0.65 ± 0.53) (Figure 3.3D). Similar trends were observed in *Morus indica* using higher rupture disc pressures in combination with a closer stopping-screen-to-target distance (Bhatnagar et al., 2002). Closer target distances and higher rupture disc pressures result in a higher microprojectile velocity, facilitating penetration of microcarriers through primary and secondary cell walls, more effectively introducing transgenes into the plant cells. A similar combination of rupture disc pressure and stopping-screen-to-target distance was found to be optimal in transient reporter expression in *Dendrobium Sonia* 17 and *Triticum* (Rasco-Gaunt et al., 1999; Tee and Maziah, 2005). Based on these results, a rupture disc pressure of 1100 psi and a stopping-screen-to-target distance of 6 cm were held constant in all subsequent experiments aimed at optimizing parameters.

3.4.3 Effect of DNA and Gold Microcarrier Concentration

In an effort to increase luciferase activity and reduce variability between bombarded calli, microcarriers were coated with either 1 µg or 2 µg of plasmid DNA, or the concentration of the gold microcarriers was increased from 60 mg/ml to 120 mg/ml. Luciferase activity in all cell lines was assayed after each bombardment. In a third set of bombardments, potential synergism between the doubling of both plasmid DNA and gold microcarriers was evaluated.

PO93X and P93AF did not demonstrate luciferase activity under any of the conditions tested (data not shown). The same general trend for both P991 and CO93D was observed as in the previous experiment where cells bombarded with 35S:LUC exhibited higher levels of luciferase activity than the respective negative control (Table 3-1). For the P991 cell line there were no statistical differences when comparing the lower and higher plasmid DNA amounts. However, increasing the concentration of gold microcarriers resulted in a trend of reduced luciferase activities (Table 3-1). For the CO93D cell line, the combination of both increased plasmid DNA and increased gold microcarrier concentration resulted in statistically higher luciferase activity when compared to the combination of lower plasmid DNA and gold concentrations. As before, variation was significant in these experiments.

These results suggest that both P991 and CO93D cell lines are capable of transient reporter expression under appropriate bombardment conditions. However, the low luciferase levels attained ($\sim < 50$ light units in most cases) are unacceptable for establishment of a reliable transformation method, despite statistical significance between those cells bombarded with 35S:LUC and those cells bombarded with the pPZP221 negative control vector (Figure 3.3 and Table 3.1). Due to these results, future optimization studies utilized 1 μ g of plasmid DNA and 60 mg/ml of gold microcarriers for P991 while further optimization with CO93D utilized 2 μ g of plasmid DNA and 120 mg/ml of gold microcarriers.

3.4.4 Effect of Multiple Bombardments

In an effort to increase transgene luciferase expression, P991 and CO93D cells were bombarded three times per plate, using 1 μ g plasmid DNA and 60 mg/ml gold

microcarrier concentration, or 2 µg plasmid DNA and 120 mg/ml gold microcarrier concentration, respectively. All cells were bombarded at an 1100 psi rupture disc pressure and a 6 cm stopping-screen-to-target distance.

Luciferase activity for both P991 and CO93D was significantly higher with multiple bombardments when compared to previous single bombardment experiments (Figure 3.4). Also, statistical significance was attained for both cell lines as compared to the negative vector controls. An increase in the number of bombardments per plate also produced an increase in transgene activity for *M. indica* by facilitating a more even spread of DNA-coated gold microcarriers over the bombarded cells (Bhatnagar et al., 2002).

3.4.5 Effect of Microcarrier Diameter

While multiple bombardments successfully reduced variation and increased transgene luciferase activity for both P991 and CO93D, further optimization was attempted by manipulating the diameter of the gold microcarriers. In addition to the 1.0 µm diameter used in all previous experiments, 1.6 µm diameter gold microcarriers were also evaluated to deliver the 35S:LUC plasmid into both P991 and CO93D. P991 cells were bombarded three times per replicate with 1 µg of DNA precipitated onto 60 mg/ml of 1.0 µm or 1.6 µm gold microcarriers. CO93D cells were bombarded three times per replicate with 2 µg of DNA precipitated onto 120 mg/ml of 1.0 µm or 1.6 µm gold microcarriers. Both cell lines were bombarded at an 1100 psi rupture disc pressure and a 6 cm stopping-screen-to-target distance.

The larger diameter gold microcarriers were more effective as vectors to deliver the DNA constructs, resulting in higher luciferase activities for both cell lines (Figure

3.5). P991 bombarded with 1.6 μm gold microcarriers displayed 350 ± 150 light units of luciferase activity compared to the 110 ± 54 light units measured when bombarded with the smaller gold microcarriers (Figure 3.5). Similarly, CO93D bombarded with the 1.6 μm gold microcarriers displayed 300 ± 92 light units of luciferase activity compared to the 190 ± 51 light units measured when bombarded with the smaller gold microcarriers (Figure 3.5). When bombarded with 1.6 μm gold microcarriers coated with 35S:LUC, P991 and CO93D cellular luciferase activity was statistically significant from respective cells bombarded with the empty vector control (Figure 3.5). This trend is in direct contrast to results presented elsewhere in the literature, where 1.0 μm gold microcarriers facilitated higher reporter gene expression (Bhatnagar et al., 2002; Tee and Maziah, 2005). This difference may be due to the size of the average *Taxus* spp. cell in culture. While most plant cells grown in culture are 20-40 μm in diameter, *Taxus* spp. cells range between 50-60 μm in diameter (Naill and Roberts, 2005). A particle with more mass may be more effective for cellular penetration, and the larger size of *Taxus* spp. cells may enable a higher tolerance for large particles.

3.4.6 Expression of Different Reporters and Promoters

Taxus cell lines P991 and CO93D possessed the capacity to express reporter genes other than firefly luciferase. In addition, promoters other than CaMV 35S were capable of driving reporter genes in *Taxus* spp. Once native GUS activity had been reduced using increased pH in the X-Gluc histochemical buffer, transgene GUS activity driven by the CaMV 35S promoter was visualized (data not shown). In addition, the reporter DsRed was capable of being expressed in both cell lines P991 and CO93D when driven by the maize ubiquitin promoter (Figure 3.6 shows results for P991; CO93D data

not shown). The rice actin promoter, however, was not functional in *Taxus* spp. (data not shown). The two promoters that were functional in *Taxus* spp., CaMV 35S and the maize ubiquitin promoter, have previously been observed to function in black pine (*Pinus nigra*), Mexican weeping pine (*P. patula*), and Mediterranean cypress (*Cupressus sempervirens*) (Grace et al., 2005; Kim et al., 2000; Lambardi et al., 1998; Nigro et al., 2004; Salaj et al., 2005).

Some promoters may be more effective at inducing gene expression in *Taxus* as the magnitude of promoter activation is highly species dependent. Therefore, the ability of *Taxus* to utilize different promoters is advantageous and can be exploited in transgene studies. For example, overexpression of a particular gene of interest may result in cell toxicity and therefore a construct utilizing a weaker promoter may be useful. On the other hand, high levels of expression of a particular regulatory gene may enhance paclitaxel accumulation and therefore a stronger promoter may be beneficial. Additionally, the use of at least two different reporter genes is necessary for the normalization of introduced transgenes under the control of different promoters. These results are therefore important in demonstrating that both different promoters and reporter genes may be used successfully in *Taxus*.

3.5 Conclusions

A reliable transient transformation system was developed with high firefly luciferase activity under control of the CaMV 35S promoter by optimizing bombardment parameters, including rupture disc pressure, stopping-screen-to-target distance, DNA plasmid concentration, gold microcarrier concentration, multiple bombardments, and microcarrier diameter. Multiple reporter genes (luciferase, GUS, and DsRed) were

expressed under a variety of promoters (CaMV 35S and maize ubiquitin). An advantage of this transient transformation system is the potential for relatively quick and efficient characterization of novel *Taxus* genes, allowing for transformation with multiple gene constructs. For example, analysis of putative paclitaxel biosynthetic pathway regulators can be achieved through co-bombardments with the transcriptional regulator and paclitaxel biosynthetic gene promoter/GUS fusion constructs (*Nims et al., manuscript in preparation*). In addition, direct localization of paclitaxel biosynthetic genes can be achieved after particle bombardment with paclitaxel biosynthetic gene/DsRed fusion constructs. These transient transformation studies can be completed within days as opposed to waiting for enough biomass for stable transformation that can take months. Before engineering of stably transformed *Taxus* cell cultures for enhanced paclitaxel accumulation can proceed (likely through *Agrobacterium*-based methods), identification and characterization of putative regulatory genes related to all facets of paclitaxel metabolism must be accomplished, and can be enabled using the transient transformation protocol presented here.

It is prudent to note, however, that while transient transformation efficiencies were optimized in this particle bombardment study, the overall transformation efficiencies may not be sufficient to affect taxane metabolism following the introduction of structural or regulatory genes related to paclitaxel biosynthesis. In a very preliminary bombardment, transient introduction of a paclitaxel-related transcription factor (Nims, unpublished data) did not cause any changes in taxane accumulation following methyl jasmonate elicitation on solid media.

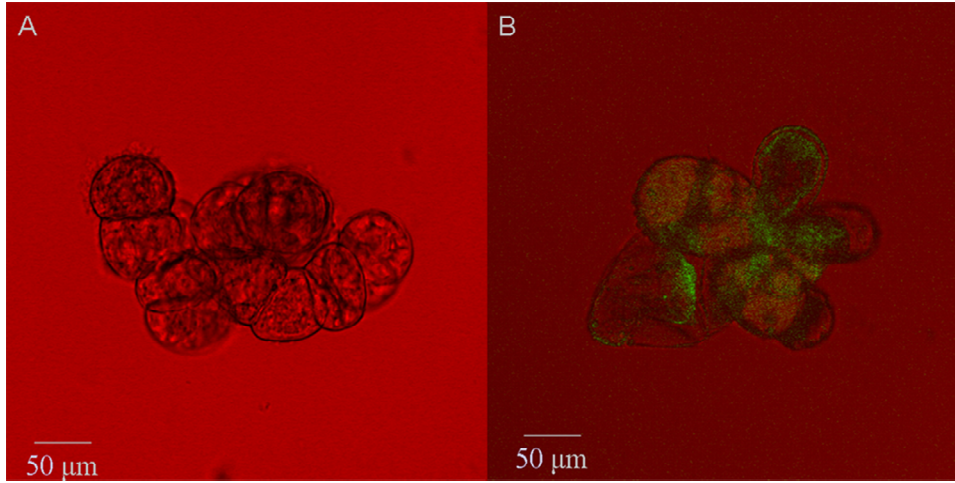


Figure 3.1: Native *Taxus* green autofluorescence

(A) *Taxus* cell line PO93X displaying no green autofluorescence. (B) *Taxus* cell line PO93X displaying green autofluorescence with spectral characteristics similar to GFP after incubation for 24 hours with *A. tumefaciens*. Cells were excited at 488 nm with an Argon laser and fluorescence was visualized with a FITC filter. Presence of autofluorescence in (B) is likely due to a native stress response.

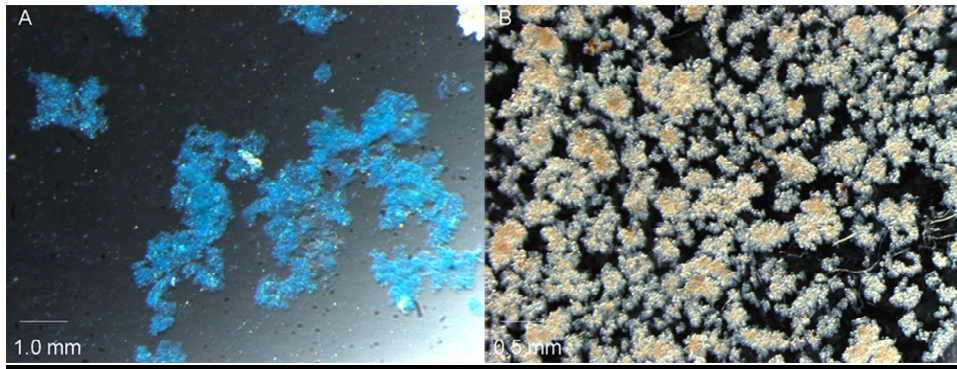


Figure 3.2: Native *Taxus* GUS activity

***Taxus* cell line P991 displaying native β -glucuronidase (GUS) activity after bombardment with PZP221 plasmid and 24 hour incubation with histochemical GUS buffer at pH 7.0 (A). *Taxus* cell line P991 displaying a reduction in native GUS activity after bombardment with PZP221 plasmid and 24 hour incubation with histochemical GUS buffer at pH 8.5 (B).**

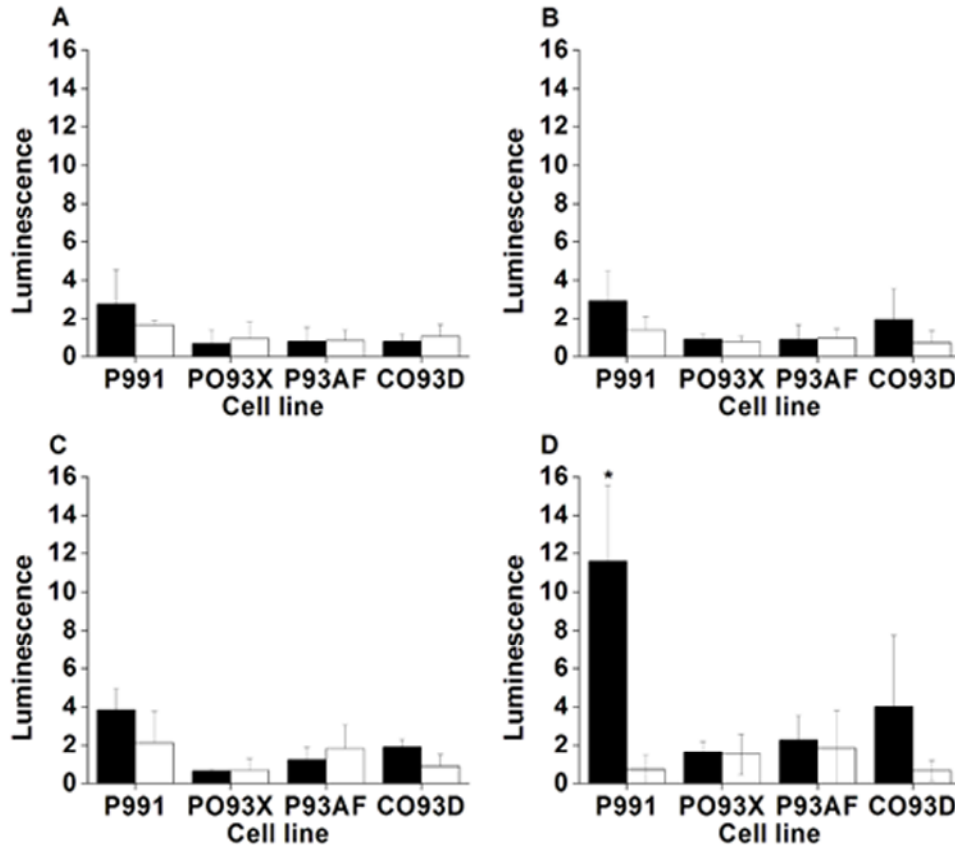


Figure 3.3: Effect of rupture disc pressure and target distance on transient transformation of four *Taxus* cell lines with either a 35S:luciferase construct or PZP221 empty vector construct

All bombardments were performed utilizing a 0.75 ml packed cell volume of *Taxus*, 1 µg of plasmid, and 60 mg/ml of 1.0 µm gold microcarriers. (A) represents a rupture disc pressure of 650 psi and a 9 cm stopping-screen-to-target distance. (B) represents a rupture disc pressure of 1100 psi and a 9 cm stopping-screen-to-target distance. (C) represents a rupture disc pressure of 650 psi and a 6 cm stopping-screen-to-target distance. (D) represents a rupture disc pressure of 1100 psi and a 6 cm stopping-screen-to-target distance. Luciferase activity is measured in light units. Individual data points represent the average of three replicates and error bars represent one standard deviation of the means. Statistical significance was determined with one-way ANOVA at a $p < 0.05$.

Table 3.1: Effect of plasmid DNA and gold microcarrier concentrations on transient luciferase activity in P991 and CO93D cell cultures. All bombardments were performed utilizing a 0.75 ml packed cell volume of *Taxus*, a 650 psi rupture disc pressure, and a 6 cm stopping-screen-to-target distance. For each data set, the first value represents the 35S:luciferase construct and the second value represents the PZP221 empty vector (i.e., negative control). Individual data points are the average of three replicates and error bars represent one standard deviation. Statistical significance between reporter construct and negative control was determined by one-way ANOVA and is indicated with a *.

P991

Gold microcarrier concentration	Plasmid DNA amount	
	1 µg	2 µg
60 mg/mL	11.58 ± 4.0 0.71 ± 0.76	25.5 ± 41.86 0.93 ± 0.70
120 mg/mL	6.15 ± 5.54 0.51 ± 0.47	4.4 ± 3.77 0.74 ± 0.13

CO93D

Gold microcarrier concentration	Plasmid DNA amount	
	1 µg	2 µg
60 mg/mL	4.01 ± 3.76 0.65 ± 0.53	55.84 ± 72.29 1.33 ± 1.38
120 mg/mL	35.41 ± 32.53 0.81 ± 0.74	14.47 ± 4.89 0.34 ± 0.14

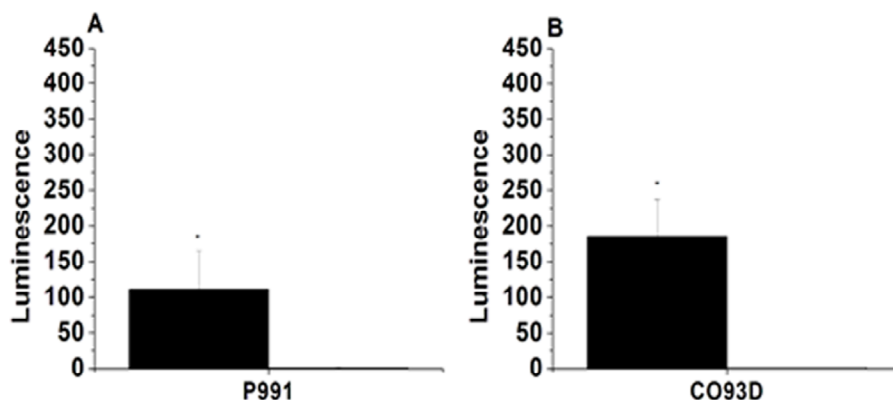


Figure 3.4: Effect of multiple bombardments on the transient transformation of *Taxus* cell lines P991 and CO93D with either a 35S:luciferase construct or PZP221 empty vector construct after three bombardments per sample

All bombardments were performed utilizing a 0.75 ml packed volume of *Taxus* at an 1100 psi rupture disc pressure and a 6 cm stopping-screen-to-target distance. (A) represents P991 bombardments with 1 µg of plasmid and 60 mg/ml of 1.0 µm gold microcarriers. (B) represents CO93D bombardments with 2 µg of plasmid and 120 mg/ml of 1.0 µm gold microcarriers. Luciferase activity is measured in light units. Individual data points represent the average of three replicates and error bars represent one standard deviation of the means. Statistical significance was determined with one-way ANOVA at a $p < 0.05$.

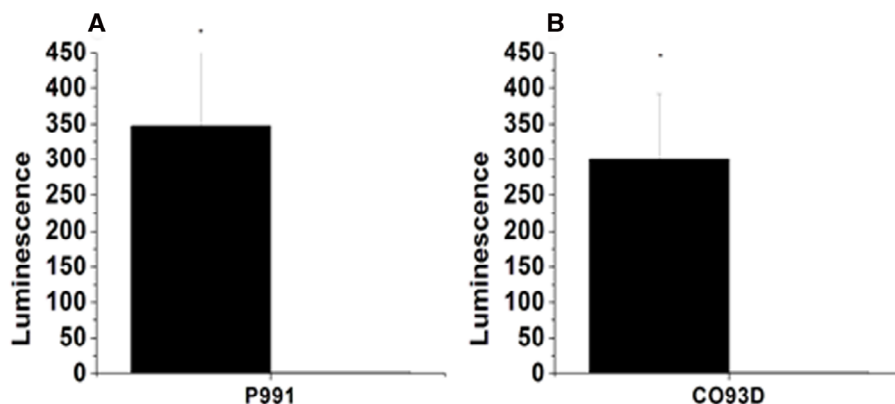


Figure 3.5: Effect of microcarrier diameter on transient transformation of *Taxus* cell lines P991 and CO93D with either a 35S:luciferase construct or PZP221 empty vector construct after three bombardments per sample

All bombardments were performed utilizing a 0.75 ml packed cell volume of *Taxus* at a 1100 psi rupture disc pressure and a 6 cm stopping-screen-to-target distance. (A) represents P991 bombardments with 1 µg of plasmid and 60 mg/ml of 1.6 µm gold microcarriers. (B) represents CO93D bombardments with 2 µg of plasmid and 120 mg/ml of 1.6 µm gold microcarriers. Luciferase activity is measured in light units. Individual data points represent the average of three replicates and error bars represent one standard deviation of the means. Statistical significance was determined with one-way ANOVA at a $p < 0.05$.

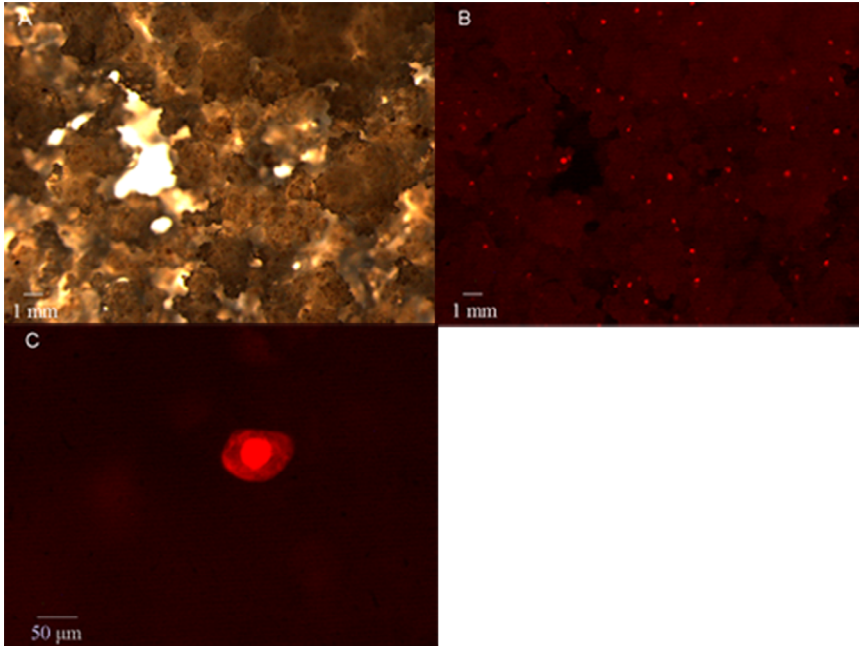


Figure 3.6: Transient transformation of two *Taxus* cell lines with a ub:dsRed construct.

Bombardments were performed utilizing 0.75 ml packed cell volume of *Taxus* at an 1100 psi rupture disc pressure and a 6 cm stopping-screen-to-target distance. Microcarriers contained 1 μ g of plasmid, and were bombarded at 60 mg/ml per bombardment. (A) represents a brightfield image of a P991 callus bombarded with UB:dsRed. (B) represents a fluorescence image of the same callus from (A), with a number of P991 cells displaying dsRed fluorescence. (C) represents a fluorescence image of single CO93D cell expressing a dsRed signal.

CHAPTER 4

AGROINFILTRATION IN NICOTIANA BENTHAMIANA AS A SYSTEM FOR THE CHARACTERIZATION OF PACLITAXEL BIOSYNTHETIC GENES

4.1 Introduction

Paclitaxel is a cyclic and poly-oxygenated diterpenoid compound containing several functional group substitutions, an oxetane ring, and a phenylisoserine C13 ester side chain. Elucidation of the paclitaxel biosynthetic pathway required a variety of technologies and approaches and represents a major accomplishment in the understanding of paclitaxel metabolism in culture. Both the cloning and functional characterization of paclitaxel biosynthetic genes were essential in identifying the numerous steps between production of the first dedicated paclitaxel precursor, taxa-4(5), 11(12)-diene, to the formation of paclitaxel.

4.1.1 Paclitaxel Biosynthesis

The first committed step in paclitaxel biosynthesis occurs after generation of geranylgeranyl diphosphate (GGPP), which is derived from three molecules of plastidial IPP and one molecule of DMAPP. The terpenoid cyclase taxadiene synthase (TASY) cyclizes the GGPP diterpenoid substrate, inducing an 1,5-hydride shift from C11 to C7 to yield taxa-4(5), 11(12)-diene (Figure 1.5) (Lin et al., 1996; Wildung and Croteau, 1996). In these *in vitro* studies, TASY also co-produces taxa-4(20), 11(12)-diene in extremely low concentrations.

The second step in paclitaxel biosynthesis is mediated by taxadiene 5 α -hydroxylase (T5 α H), a cytochrome P450 (Jennewein et al., 2004). T5 α H causes an unusual, though not unprecedented, hydroxylation at the C5 position of the parental olefin taxa-4(5), 11(12)-diene, yielding taxa-4(20),11(12)-dien-5 α -ol (Figure 1.5). Interestingly, T5 α H also utilized the synthetic substrate taxa-4(20), 11(12)-diene to produce taxa-4(20),11(12)-dien-5 α -ol, initially suggesting that allylic rearrangement of the double bond from the 4(5) position to the 4(20) position may not be mediated by T5 α H, but by a potential non-P450 taxadiene isomerase. However, in a study utilizing *Taxus* cell microsomes inhibited in P450 activity, no isomerization of taxa-4(5), 11(12)-diene was observed, suggesting that migration of the double bond from 4(5) to 4(20) was an inherent feature of T5 α H activity and that the taxane P450 could utilize multiple substrates (Jennewein et al., 2004).

During the second stage of paclitaxel biosynthesis, taxa-4(20),11(12)-dien-5 α -ol undergoes a series of P450-mediated hydroxylations and a single acylation, followed by oxidation at C9 and epoxidation at the C4 C5 double bond. The order of these CYP450-mediated oxygenations is not known with certainty, though a survey of oxygenation frequency in cell culture-derived taxanes tentatively indicates an order proceeding from C5, to C10, C2, C9, C13, C7, and finally at the C1 position (Croteau, 2006; Jennewein and Croteau, 2001; Ketchum, 2006). The single acylation step is considered to reside within this litany of oxygenations, with initial work indicating that it occurs early in the pathway. Completion of this second stage yields a hypothetical taxane intermediate with a structure similar to 10-deacetyl-2-debenzoylbaccatin III (Figure 1.5).

From the known genes that have been cloned and characterized, it appears that both T13 α H and TDAT (hydroxylates C5 and acylates the oxygen on C5, respectively) utilize taxa-4(20),11(12)-dien-5 α -ol as a substrate (Figure 1.5) (Jennewein et al., 2001; Walker et al., 1999). T13 α H is responsible for the conversion of taxa-4(20),11(12)-dien-5 α -ol to taxa-4(20),11(12)-diene-5 α ,13 α -diol, and TDAT catalyzes the acylation of taxa-4(20),11(12)-dien-5 α -ol to taxa-4(20),11(12)-dien-5 α -yl-acetate. Additionally, both T13 α H and TDAT do not catalyze reactions with more advanced taxane intermediates, indicating that these decorations occur relatively early in paclitaxel biosynthesis (Jennewein et al., 2001; Walker et al., 1999).

While genes were isolated from these cDNA libraries encoding T5 α H, T13 α H, and T10 β H, these methyl jasmonate-induced *T. cuspidata* cDNA libraries also yielded a number of novel CYP450 oxygenases that were related to known taxane CYP450s. Incubating these novel CYP450s with synthetic taxane metabolites decorated at select core locations lead to the discovery of taxane 2 α -hydroxylase (T2 α H) and taxane 7 β -hydroxylase (T7 β H).

The genes responsible for the remaining oxygenation steps (C1, C4, and C9), including oxetane ring formation, remain to be cloned and enzymatically characterized. However, following oxygenation of the core taxane ring, acylation of the C5 position, and epoxide ring formation, a hypothetical intermediate with a structure similar to 10-deacetyl-2-debenzoylbaccatin III is generated, and likely utilized in the final stage of paclitaxel biosynthesis (Figure 1.6).

This hypothetical poly-hydroxylated taxane metabolite is hypothesized to undergo acylation of the 2 α hydroxyl group via 2 α -hydroxytaxane-2-O-benzoyltransferase (DBT),

yielding 10-DAB (Figure 1.6) (Walker and Croteau, 2000b). The failure of DBT to utilize simpler taxane intermediates, such as taxa-4(20), 11(12)-dien-2 α , 5 α -diol, in conjunction with the high regioselectivity of this enzyme to only acylate the 2 α hydroxyl group, suggested that this second acylation step of the core taxane ring occurs after multiple oxygenation of the core taxane ring.

Following generation of 10-DAB, 10-deacetylbaccatin-III-10-O-acetyl transferase (DBAT) acylates the hydroxyl group at the C10 position, yielding baccatin III (Figure 1.6) (Menhard, 1998; Walker and Croteau, 2000a). Like DBT, the DBAT-mediated acylation occurs with a high degree of regioselectivity. DBAT does not acylate the 1 β , 7 β , or 13 α hydroxyl groups of 10-DAB, nor does it acylate the 5 α hydroxyl group of taxa-4(20), 11(12)-dien-5 α -ol.

Baccatin III is the last presumed non-side chain-containing taxane that participates in paclitaxel biosynthesis. The enzyme baccatin III:3-amino-3-phenylpropionyltransferase (BAPT) catalyzes conjugation of the β -phenylalanoyl-CoA side chain only to the C13 hydroxyl group of baccatin III to generate 3'-N-debenzoyl-2'-deoxytaxol (Figure 1.6) (Walker et al., 2002b). The β -phenylalanoyl-CoA side chain is derived from α -phenylalanine, where phenylalanine aminomutase (PAM) causes an intermolecular transfer of the pro-3S hydrogen to the C2' position of phenylalanine, incurring a nitrogen shift from C2' to C3' (Figure 1.6) (Steele et al., 2005; Walker et al., 2004).

Following 3'-N-debenzoyl-2'-deoxytaxol formation, three further steps must occur before paclitaxel synthesis is complete. These include side chain C2' hydroxylation and N-benzoylation of the C3' position (Long and Croteau, 2005; Long et

al., 2008). Earlier studies were ambiguous regarding the timing of C2' hydroxylation, with the data failing to discriminate between C2' hydroxylation preceding or succeeding side chain conjugation to the decorated core taxane ring. However, recent studies demonstrated failure of a *Taxus* cell-free enzyme system to hydroxylate β -phenylalanine and 2'-deoxytaxol, providing strong evidence that the hydroxylation event occurs after side chain conjugation on route to paclitaxel formation (Long and Croteau, 2005).

Paclitaxel is formed when 3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase (DBTNBT) catalyzes the benzamidation of 3'-N-debenzoyl-2'-deoxytaxol with benzoyl-CoA to yield paclitaxel (Figure 1.6). DBTNBT is regiospecific for acylation at the 3' amino group of the paclitaxel side chain, and is incapable of acylating the side chain alone (Long and Croteau, 2005; Walker et al., 2000; Walker et al., 2002a).

4.1.2 Established Methods used to Characterize Taxane Biosynthetic Genes

Cloning of paclitaxel biosynthetic genes provided a large amount of nucleotide sequence information. While bioinformatic analyses of nucleotide sequence may offer limited insight into enzyme activity, functional characterization is necessary to confirm the function of these genes predicted *in silico*. This is especially relevant for the taxane CYP450 family of heme-containing, NADPH-dependent enzymes. Similar to other plant CYP450 proteins, nucleotide sequence information derived from taxane CYP450s provided little or no information regarding their *in vivo* function and thus necessitates biochemical characterization (Chapple, 1998).

Cell-free *in vitro* enzyme assays, utilizing either *Escherichia coli* or *Spodoptera frugiperda* insect cell microsomes as protein production engines, are model systems that have been used to functionally characterize genes participating in paclitaxel biosynthesis

(Chau et al., 2004; Loncaric et al., 2006). Generally adopted due to the ease of growth and transformation, *E. coli* and *S. frugiperda* insect cell microsomes were successful in ascertaining the function of some taxane structural genes. However, the functional accuracy of these cell-free *in vitro* systems is compromised by limitations in substrate availability and artifacts in product generation. In particular, the *E. coli* and *S. frugiperda* systems are prone to host protein contamination despite stringent attempts at isolation of a pure target protein that may produce *in vitro* artifacts following incubation with a substrate, thereby producing metabolic products not directly related to the native function of the studied gene (Jennewein et al., 2001).

Additional studies examined the expression of paclitaxel biosynthetic genes in microbial systems, such as *E. coli* or *Saccharomyces cerevisiae*. While the first two steps in the paclitaxel pathway were demonstrated in *E. coli*, manipulation of the *Taxus* genes was required for proper protein function (Ajikumar et al., 2010). For example, for proper TASY function, the plant transit peptide must be truncated in order to prevent microbial targeting to the bacterial cell wall where it would not be functional. Thus, expression in *E. coli* is not ideal, due to additional gene manipulations that are required. Additional attempts to address the limitations of cell-free systems in *S. cerevisiae* demonstrated that native yeast CYP450 reductases were not sufficient to permit proper taxane CYP450 hydroxylase function (Jennewein et al., 2005). Subsequent work partially addressed this limitation through the creation of the WAT11 *S. cerevisiae* strain. The WAT11 *S. cerevisiae* strain co-expresses an *Arabidopsis thaliana* CYP94A1 reductase and takes advantage of the plastic interaction between *Taxus* CYP450 hydroxylases and the *A. thaliana* CYP94A1 reductase, permitting sufficient electron coupling to facilitate an

enzymatic reaction in the presence of a proper substrate (Jennewein et al., 2001). While the WAT11 strain was utilized in the characterization of T2 α H and T7 β H (Chau and Croteau, 2004; Chau et al., 2004), these studies would not have been possible if not for the use (+)-taxusin, a non-commercially available taxane substrate that is either synthesized or purified from *Taxus* bark (Chau and Croteau, 2004; Chau et al., 2004; Jennewein et al., 2001; Schoendorf et al., 2001). Utilization of (+)-taxusin, a surrogate taxane compound that does not directly contribute to paclitaxel formation, exploits inherent substrate plasticity common to the taxane CYP450 proteins, allowing for the examination of taxane CYP450 protein function in the absence of native substrates. Due to difficulties in (+)-taxusin purification or harvest, widespread use of this taxane substrate is not likely in future characterization studies of novel taxane CYP450s hydroxylases. Thus, the limitation of substrate availability in the WAT11 system is similar to the limitation of substrate availability in cell-free *in vitro* systems.

4.1.3 Transient Transformation in Plants as a Protein Characterization System

The obligate co-expression of an *A. thaliana* CYP450 reductase for CYP450 hydroxylase activation in the WAT11 *S. cerevisiae* system and the requirement of a surrogate taxane substrate illustrate the major limitations of plant enzyme characterization in microbial systems (Jennewein et al., 2005). This difficulty in plant CYP450 hydroxylase expression in microbial systems is well described in the literature. For example, expression of the plant CYP450 hydroxylase, F3'5'H, in *E. coli* required the generation of a fusion protein with a plant P450 reductase to successfully co-express F3'5'H in the microbial host (Leonard et al., 2006). Additionally, expression of a novel CYP71AV1 from *Asterias annua* in *S. cerevisiae* required the co-expression of a

CYP450 reductase also isolated from *A. annua* to ensure proper function (Ro et al., 2006). In addition to CYP450 reductases, other plant accessory proteins may be necessary for accurate enzymatic activity; chaperones related to protein stability present another example of plant-specific proteins that may contribute both to the accurate and successful characterization of plant enzymatic proteins.

The difficulties encountered in characterizing plant proteins in microbial systems suggest that plant systems may be more suitable for the expression of more problematic plant proteins than microbial systems, due to the likely presence of plant-specific accessory proteins (Yajun et al., 2005). The adoption of a plant protein expression platform must focus on transient transformation and not stable transformation, due to the time commitments associated with each. Stable transformation in plants is a time-consuming process (months) relative to transient plant transformation, often requiring physical manipulation that is not easily automated (Gelvin, 2009; Jones and Sparks, 2009). Even in cases where stable transformation is successful, negative pleiotropic effects may preclude the use of the transgenic plant material, as demonstrated by the dwarf *A. thaliana* plants stably expressing TASY (Besumbes et al., 2004). Additionally, the extended time required to produce stable plant transformants is compounded by the characterization of genes related to paclitaxel biosynthesis. Several genes remained uncloned and uncharacterized from the paclitaxel biosynthetic pathway, including those taxane CYP450s that oxygenate the taxane skeleton at C1, C4, and C9. Functional characterization of these genes may require advanced taxane substrates that are neither commercially available nor simple to synthesize (e.g., (+)-taxusin), thus requiring the assembly of multi-step recombinant pathways *in planta* when substrates are not available.

Generation of multi-gene constructs is an unwieldy process that decreases *Agrobacterium*-mediated stable transformation efficiency due to the increased size of appropriate T-DNA cassettes, and the successive transformation of individual pathway genes into a plant host requires even greater time investments than regeneration from a single transgenic event (Sheludko, 2008).

4.1.4 Agroinfiltration in *Nicotiana benthamiana*

Agroinfiltration in *Nicotiana benthamiana* leaves is a simple and effective method to transiently characterize protein function. Compared to the utilization of microbial or stable plant transformation systems to characterize protein function, agroinfiltration in *N. benthamiana* has several advantages.

Compared to microbial systems, transient expression of genes following agroinfiltration into *N. benthamiana* allows for evaluation of protein function, particularly CYP450 hydroxylases, in near-native environments that do not require additional modification to the target genes and where plant-specific accessory proteins such as chaperones or CYP450 reductases, are likely to be present. Thus, this potentially precludes the need to co-transform plant-specific accessory proteins that may be necessary for proper protein function. Due to this factor alone, if successfully implemented, agroinfiltration in *N. benthamiana* may provide a desirable alternative to *E. coli*, *S. cerevisiae*, and *S. frugidera* insect cell microsome systems. Additionally, agroinfiltration in *N. benthamiana* also potentially addresses the limitation of substrate availability. This will be discussed in the next paragraph.

Transient gene expression following agroinfiltration into *N. benthamiana* possesses several advantages over stable plant transformation systems to characterize

protein function. In contrast to the lengthy time commitment usually associated with the production of stable transformants (months), agroinfiltration is a transient transformation method in plants leaves that requires considerably less time (days) than stable transformation methods (Wydro et al., 2006). Additionally, agroinfiltration in *N. benthamiana* may be utilized to introduce more than one T-DNA from different *Agrobacterium* cells containing different binary vectors, effectively permitting the transient reconstruction of non-native metabolic pathways to produce substrates. In regard to the characterization of metabolite biosynthetic genes, this may preclude the need for non-commercially available substrates required for characterization of protein function, due to the potential to transiently produce these compounds in *N. benthamiana* following co-agroinfiltration of multiple genes in different *Agrobacterium*. This stacking of multiple genes without the generation of large and complicated constructs was successfully demonstrated in the production of long-chain polyunsaturated fatty acids (Wood et al., 2009).

Agroinfiltration in *N. benthamiana* is an established system, already used for the production of antibodies and proteins for use in therapeutic treatments (D'Aoust et al., 2009; Fischer et al., 1999; Sheludko, 2008). Furthermore, agroinfiltration in *N. benthamiana* has been utilized as a tool in the study of VIGS (virus-induced gene silencing), *in vivo* studies of promoter /transcription factor interactions, and the effects of differential protein targeting (Constantin et al., 2008; Di Fiore et al., 2002; Sawers et al., 2006; Yang et al., 2000). In a study examining the plant photosynthetic protein machinery, *N. benthamiana* agroinfiltration was utilized as a novel system to characterize *Zea maize* Mg⁺ chelatase function *in planta* (Sawers et al., 2006).

4.1.5 Agroinfiltration in *N. benthamiana* for Taxane Accumulation

The successful stable transformation of *A. thaliana* and *Lycopersicon esculentum* with taxadiene synthase (TASY) demonstrated that native plant GGPP pools are sufficient to drive metabolic flux into a non-native metabolic pathway and provided initial evidence that plant systems can be utilized as heterologous protein expression platforms for genes related to taxane metabolism (Besumbes et al., 2004; Kovacs et al., 2007). These papers also demonstrated the benefits of utilizing a transient transformation system in order to express genes related to paclitaxel metabolism, as negative pleiotropic phenotypes were observed following the expression of a taxane biosynthetic gene (Besumbes et al., 2004). While these negative pleiotropic phenotypes were addressed through the use of an inducible promoter (*A. thaliana*) or a carotenoid-deficient mutant (*L. esculentum*), these stable transformation methods still require multiple months to regenerate positive transformants, a disadvantage not seen in transient plant transformation systems such as agroinfiltration in *N. benthamiana*.

Development of an agroinfiltration system in *N. benthamiana* to characterize taxane biosynthetic gene function requires several proof-of-concept demonstrations. First, a method must be developed for the extraction of early stage taxanes. Second, accumulation of taxadiene, the first dedicated substrate of paclitaxel metabolism, must be demonstrated following agroinfiltration of TASY into *N. benthamiana*. Third, the stacking capacity and functional expression of co-agroinfiltration must be demonstrated following agroinfiltration of both TASY and T5 α H (the first and second genes in paclitaxel biosynthesis, respectively) to yield taxa-4(20),11(12)-dien-5 α -ol (Figure 1.5). The prospective applications of this method may be useful, as characterization of novel

taxane CYP450 proteins would likely require native plant proteins that would generally be present in *N. benthamiana*. Additionally, the potential ease by which to transiently recreate non-native metabolic pathways through co-agroinfiltration gene stacking in *N. benthamiana* may preclude the need for non-commercially available substrates that are currently required for taxane CYP450 protein characterization.

4.2 Materials and Methods

A complete description of the materials and methods is located in Chapter 2. Of particular interest are binary plant expression vectors (2.4.3), agroinfiltration (2.5.3), and biochemical analyses through Solid Phase Extraction (SPE) and GC-MS (2.3.3).

4.3 Results

4.3.1 Agroinfiltration as a Method to Deliver Transgenes into *N. benthamiana* Tissue

Agroinfiltration into a *N. benthamiana* leaf approximately three inches long with *Agrobacterium* strain C56C1 alone yielded no GUS activity, while GUS activity was observed in *N. benthamiana* leaf tissue following agroinfiltration with C56C1 containing pCAMBIA1301 (35S:intron-GUS cassette) (Figure 4.1A and Figure 4.1B, respectively). GUS activity was observed following an overnight incubation in GUS buffer containing the X-Gluc substrate at 37°C. The microwound created by the razor blade is very prominent in the images, and transformation efficiency appears to be approximately 30%. Additionally, the co-agroinfiltration of multiple reporter genes into *N. benthamiana* leaf parenchyma cells demonstrates that multiple transformation events, and thus stacking of genes, are possible in the *N. benthamiana* agroinfiltration system (Figure 4.2).

4.3.2 Nonadecane Recovery from a Flow-Through Solid Phase Extraction Column

Prior to agroinfiltration of TASY into *N. benthamiana* and recovery of taxa-4(5), 11(12)-diene from plant tissue, a suitable recovery method was needed to extract and purify target taxanes, increasing resolution of sample analysis through the reduction of co-eluting compounds during GC separation preceding MS analysis. Unfortunately, taxa-4(5), 11(12)-diene is not commercially available as a standard to use in extraction/purification studies. Therefore, a surrogate substrate was required for the analysis of chemical recovery. Nonadecane, another undecorated 20-unit hydrocarbon, was utilized to test the efficacy of a modified silica/magnesium sulfate SPE column (Ketchum, 2006). Nonadecane has previously been utilized as an internal standard in the quantification of taxa-4(5), 11(12)-diene, as the lack of functional group decorations permits it to behave in a manner chemically similar to taxa-4(5), 11(12)-diene during SPE sample preparation (Besumbes et al., 2004).

Following SPE purification of 10 pg/μl of a nonadecane sample and GC-MS analysis, a distinct nonadecane peak is seen at 17.4 minutes (Figure 4.3A). Select ion monitoring (SIM) of this peak confirms the presence of all corresponding nonadecane ion fragments, identifying this peak at 17.4 minutes as nonadecane (Figure 4.3B). Injection of solvent alone showed no distinct peak at 17.4 minutes (data not shown), further confirming that the compound recovered in spiked samples at 17.4 minutes is true nonadecane and not a contaminant compound. Quantification of nonadecane recovery after SPE purification compared to an injection of pure 10 pg/μl nonadecane standard was 90%, demonstrating that the silica sorbent of the SPE column has minimal affinity for undecorated 20-unit hydrocarbon chains, and suggesting that it will not inadvertently

decrease taxa-4(5), 11(12)-diene or nonadecane concentrations in future *N. benthamiana* leaf samples. Additionally, comparison of nonadecane-spiked (10 ng/μl) *N. benthamiana* samples following SPE extraction (nonadecane was added after SPE extraction of *N. benthamiana* tissue) demonstrates that nonadecane is recoverable following addition to *N. benthamiana* leaf tissue and that SPE is effective in removing contaminant compounds from *N. benthamiana* leaf tissue that may otherwise reduce sensitivity of detection (Figure 4.4A and Figure 4.4B). Nonadecane from spiked *N. benthamiana* tissue was not quantified, as recovery was sufficiently high from the nonadecane-only sample. Additionally, future agroinfiltration with TASY would focus primarily on the qualitative, not quantitative, detection of a taxane product.

4.3.3 Taxa-4(5), 11(12)-diene Accumulation in *N. benthamiana* Following Agroinfiltration with Taxadiene Synthase

Previous work demonstrated that native plant GGPP pools are sufficient to allow for taxa-4(5), 11(12)-diene accumulation in transgenic *A. thaliana* and *L. esculentum* constitutively expressing TASY (Besumbes et al., 2004; Kovacs et al., 2007). Thus, agroinfiltration of 35S:TASY into *N. benthamiana* tissue is expected to yield taxa-4(5), 11(12)-diene. GC-MS analysis of *N. benthamiana* leaf material (4 g fresh weight, 5 days post-agroinfiltration) following agroinfiltration with 35S:TASY displayed a distinct peak with a retention time of 19.7 minutes (Figure 4.5A). SIM of this peak revealed the presence of all published and corresponding taxa-4(5), 11(12)-diene fragmentation ions at this retention time, confirming this peak as authentic taxa-4(5), 11(12)-diene (Figure 4.5B). *N. benthamiana* samples agroinfiltrated with 35S:T5αH as a negative control showed no distinct peak at 19.7 minutes (Figure 4.6A). Additionally, in samples

agroinfiltrated with 35S:T5 α H, neither the parent ion for taxa-4(5), 11(12)-diene (272 m/z) (Figure 4.5B) nor 4(20),11(12)-dien-5 α -ol (288 m/z) (Figure 4.6C) were detected. Taken in total, the peak at 19.7 minutes represents taxa-4(5), 11(12)-diene accumulation following agroinfiltration of 35S:TASY, resulting from TASY activity. Taxa-4(5), 11(12)-diene was not quantified, as the primary goal of this agroinfiltration was qualitative detection of a taxane metabolite product and not quantification.

4.3.4 Co-agroinfiltration of TASY and T5 α H Leads to the Absence of Taxa-4(5), 11(12)-diene and Taxa-4(20),11(12)-dien-5 α -ol

It was initially expected that co-agroinfiltration of 35S:TASY and 35S:T5 α H would yield taxa-4(20), 11(12)-dien-5 α -ol, due to the accumulation of taxa-4(5), 11(12)-diene following agroinfiltration of 35S:TASY and previous work demonstrating the capacity of agroinfiltration in *N. benthamiana* to introduce multiple genes following co-agroinfiltration with different constructs (Sawers et al., 2006). GC-MS analysis of *N. benthamiana* leaf material (4 g fresh weight, 5 days post-agroinfiltration) following co-agroinfiltration with 35S:TASY and 35S:T5 α H showed no discernable peaks in the analyzed SPE-prepared samples, except for the spiked nonadecane peak (Figure 4.7A). GC-MS analysis also confirmed the lack of taxa-4(5), 11(12)-diene (272 m/z) (Figure 4.7B) and 4(20),11(12)-dien-5 α -ol (288 m/z) (Figure 4.7C) parent ions.

There exists three possibilities that may explain the lack of both taxa-4(5), 11(12)-diene and taxa-4(20), 11(12)-dien-5 α -ol in *N. benthamiana* leaf tissue co-agroinfiltrated with 35S:TASY and 35S:T5 α H: 1) Native *N. benthamiana* proteins are converting both taxa-4(5), 11(12)-diene and taxa-4(20), 11(12)-dien-5 α -ol into novel products; 2) The co-agroinfiltration is failing to co-transform the same *N. benthamiana* cells; and 3) That

T5 α H is not functioning as previously described in the literature (Jennewein et al., 2004). The first possibility can be immediately eliminated, as taxa-4(5), 11(12)-diene was detected following agroinfiltration with 35S:TASY (Figure 4.5). This, however, does not preclude the other two possibilities.

4.3.5 Determination that Co-agroinfiltration is not Failing to Co-transform the Same Cells in *N. benthamiana*

To exclude the possibility of an unsuccessful co-agroinfiltration with 35S:TASY and another *Agrobacterium* containing another binary vector, 35S:TASY and 35S:mCherry were co-agroinfiltrated into *N. benthamiana* leaf tissue to demonstrate activity of both constructs introduced through co-agroinfiltration. 4 g fresh weight samples collected 5 days post-agroinfiltration displayed both mCherry fluorescence and taxa-4(5), 11(12)-diene accumulation, indicating the successful T-DNA transfer of both transgenes into *N. benthamiana* mesophyll cells. Most importantly, this result demonstrates the successful accumulation of taxa-4(5), 11(12)-diene when co-agroinfiltrated with another transgene, eliminating unsuccessful co-agroinfiltration as a possibility (Figure 4.8A and Figure 4.8B). Additionally, a previous experiment co-agroinfiltrated with various fluorescent reporter genes also suggests that co-agroinfiltration is capable of introducing multiple T-DNAs into the same *N. benthamiana* cell (Figure 4.2).

4.3.6 Determination that T5 α H is Utilizing Taxa-4(5), 11(12)-diene as a Substrate Following Co-agroinfiltration of TASY and T5 α H

The absence of both taxa-4(5), 11(12)-diene and taxa-4(20),11(12)-dien-5 α -ol when 35S:TASY and 35S:T5 α H are co-agroinfiltrated into *N. benthamiana* and the

presence of taxa-4(5), 11(12)-diene when 35S:TASY and 35S:mCherry are co-agroinfiltrated into *N. benthamiana* leaf tissue suggests two possibilities. First, T5 α H may indeed be metabolically active and converting taxa-4(5), 11(12)-diene into a novel and undetected product with near 100% conversion efficiency. The second possibility is that the currently-utilized extraction/purification method may not be sufficient to extract the product of T5 α H.

Interestingly, when both TASY and T5 α H are stably expressed in *N. sylvestris* trichomes, neither taxa-4(5), 11(12)-diene nor taxa-4(20), 11(12)-dien-5 α -ol are detected. However, a novel taxane not previously detected in any *Taxus* system, 5(12)-oxa-3(11)-cyclotaxane, was detected (Figure 4.9A and Figure 4.9B), suggesting that T5 α H may catalyze another reaction beyond what is expected (Rontein et al., 2008). However, SIM analysis for the 288 m/z parent ion of 5(12)-oxa-3(11)-cyclotaxane in the *N. benthamiana* TASY and T5 α H co-agroinfiltrated samples here did not yield a detectable peak (Figure 4.9C).

Despite the inability to detect taxa-4(20), 11(12)-dien-5 α -ol or the 5(12)-oxa-3(11)-cyclotaxane in samples co-agroinfiltrated 35S:TASY and 35S:T5 α H, the absence of taxa-4(5), 11(12)-diene strongly suggests that T5 α H is utilizing it as a substrate. Previous *in vitro* assays have determined that the reaction catalyzed by T5 α H can be an efficient one, often utilizing all available substrate (Hefner et al., 1996; Jennewein et al., 2004). Thus, T5 α H may be utilizing all taxa-4(5), 11(12)-diene in *N. benthamiana* tissue co-agroinfiltrated with 35S:TASY and 35S:T5 α H. Assuming equal T-DNA transfer of 35S:TASY and 35S:T5 α H following co-agroinfiltration, a decrease in the abundance of 35S:T5 α H may allow detection of taxa-4(5), 11(12)-diene and provide indirect evidence

that T5 α H is utilizing it as a substrate. Agroinfiltration of 35S:TASY and 35S:T5 α H into *N. benthamiana* at a ratio of 9:1 (volume:volume) (TASY and T5 α H, respectively) leads to the GC-MS detection of a discrete taxa-4(5), 11(12)-diene peak at a retention time of 19.7 minutes (in 4 g of *N. benthamiana* leaf tissue collected 5 days post agroinfiltration) (Figure 4.10A). This retention time is identical to the detected taxa-4(5), 11(12)-diene peak when 35S:TASY was agroinfiltrated alone into *N. benthamiana* leaf tissue (Figure 4.5A). SIM confirms the presence of all corresponding taxa-4(5), 11(12)-diene fragmentation ions, identifying that this peak is authentic taxa-4(5), 11(12)-diene (Figure 4.10B).

Additional experimental evidence supporting the use of taxa-4(5), 11(12)-diene by T5 α H following co-agroinfiltration with 35S:TASY and 35S:T5 α H can be demonstrated by comparing taxa-4(5), 11(12)-diene accumulation following co-agroinfiltration of either 35S:TASY and 35S:T5 α H or 35S:TASY and 35S:mCherry, both at 9:1 ratios. If T5 α H is converting taxa-4(5), 11(12)-diene into a novel and undetectable product following co-agroinfiltration in *N. benthamiana* tissue, then it is plausible that taxa-4(5), 11(12)-diene abundance will be higher in samples co-agroinfiltrated with 35S:TASY and 35S:mCherry than samples co-agroinfiltrated with 35S:TASY and 35S:T5 α H. When 35S:TASY and 35S:mCherry are agroinfiltrated into *N. benthamiana* samples at a ratio of 9:1, a distinct taxa-4(5), 11(12)-diene is observed at 19.7 minutes (in 4 g of *N. benthamiana* leaf tissue collected 5 days post agroinfiltration) (Figure 4.11A). SIM analysis confirms the identify of this peak as taxa-4(20), 11(12)-diene (Figure 4.11B). Quantification of taxa-4(5), 11(12)-diene in both types of agroinfiltration samples demonstrated that taxa-4(5), 11(12)-diene concentration accumulates to $3.27 \pm$

0.13 $\mu\text{g/g}$ in 35S:TASY/35S:mCherry co-agroinfiltrated samples and $0.56 \pm 0.1 \mu\text{g/g}$ in 35S:TASY/35S:T5 α H co-agroinfiltrated samples (Figure 4.12). This represents quantitative evidence that the presence of T5 α H in the co-agroinfiltration impacts taxa-4(5), 11(12)-diene concentrations, where a higher abundance of T5 α H relative to TASY leads to the eventual conversion of taxa-4(5), 11(12)-diene to a novel and undetected compound.

4.4 Discussion and Conclusions

4.4.1 Accumulation of Taxa-4(5), 11(12)-diene Following Agroinfiltration with TASY in *N. benthamiana*

A reproducible *N. benthamiana* agroinfiltration system was developed for the agroinfiltration of taxane structural biosynthetic genes. Reliable production of taxa-4(5), 11(12)-diene as the result of TASY agroinfiltration was demonstrated. The accumulation of taxa-4(5), 11(12)-diene in TASY agroinfiltrated *N. benthamiana* leaf material supports previous data suggesting that native plant GGPP pools are sufficient to drive metabolism into taxane biosynthesis (Besumbes et al., 2004; Kovacs et al., 2007). Due to its transient nature and the use of already developed tissue, agroinfiltration of TASY into *N. benthamiana* mesophyll cells did not induce a detrimental phenotype as observed in *A. thaliana* stably transformed with TASY (Besumbes et al., 2004). Additionally, chlorosis was not reliably associated with TASY overexpression (Besumbes et al., 2004), as *N. benthamiana* leaves agroinfiltrated with both TASY, T5 α H, and control *Agrobacterium* inconsistently displayed chlorotic regions 3-5 days after agroinfiltration, likely as a result of gradual cell death following agroinfiltration with *A. tumefaciens*.

Direct comparison of taxa-4(5), 11(12)-diene accumulation in *N. benthamiana* following transient co-agroinfiltration of 35S:TASY and 35S:mCherry at a 9:1 ratio (3.2 µg/g of fresh weight) to other plant systems that have been engineered to accumulate taxa-4(5), 11(12)-diene is difficult, as both *A. thaliana* and *L. esculentum* were stably transformed with TASY only and that different tissues were measured. Taxa-4(5), 11(12)-diene in *A. thaliana* measured approximately 600 ng/g of dry weight, while accumulation in *L. esculentum* measured 16 µg/g of freeze dried fruit (Besumbes et al., 2004; Kovacs et al., 2007).

4.4.2 Conversion of Taxa-4(5), 11(12)-diene into a Novel Product by T5αH Following Co-agroinfiltration of TASY and T5αH

Following co-agroinfiltration with both 35S:TASY and 35S:T5αH, neither taxa-4(5), 11(12)-diene nor taxa-4(20),11(12)-dien-5α-ol was observed. Successful detection of taxa-4(5), 11(12)-diene using GC-MS analysis was achieved following the co-agroinfiltration of 35S:TASY and 35S:T5αH at a 9:1 ratio. Additionally, the concentration of taxa-4(5), 11(12)-diene is reduced in co-agroinfiltrated samples containing 35S:TASY and 35S:T5αH when compared to co-agroinfiltrated samples containing 35S:TASY and 35S:mCherry. Manipulating TASY and T5αH ratios within the co-agroinfiltration effectively amounted to a dosage effect, where delivery of *Agrobacterium* containing the T5αH transgene is less than *Agrobacterium* containing the TASY transgene due to the 9:1 (TASY:T5αH) ratio. This enabled detection of taxa-4(5), 11(12)-diene, due to the reduced presence of T5αH.

Detection of taxa-4(5), 11(12)-diene following co-agroinfiltration of 35S:TASY and 35S:T5αH at a ratio of 9:1 suggests that T5αH is utilizing taxa-4(5), 11(12)-diene as

a substrate. However, the specific nature of the reaction catalyzed by T5 α H remains unknown. Previous expression of TASY and T5 α H in *N. sylvestris* lead to the detection of a novel taxane, 5(12)-oxa-3(11)-cyclotaxane, while neither taxa-4(5), 11(12)-diene nor taxa-4(20), 11(12)-dien-5 α -ol was not detected (Rontein et al., 2008). This suggests that T5 α H may be catalyzing different reactions than previously described (Jennewein et al., 2004). T5 α H belongs to the CYP450 enzyme super-family, a group of catalytic proteins well regarded as possessing significant plasticity in both substrate utilization and product generation (Bennetzen et al., 2009; Chapple, 1998). For example, CYP75A 3'5'H flavonoid hydroxylase from *Catharanthus roseus* generated both the expected 3'5' hydroxylated product in addition to an unexpected 3' hydroxylated product in *in vitro* assays (Kaltenbach et al., 1999). Furthermore, CYP80 (berbamunine synthase) from *Berberis stolonifera* demonstrated a capacity to generate multiple products, as it is capable of producing either (*R,S*)-berbamunine, guattegaumerine, or 2'-norberbamunine when presented with the appropriate substrate (Stadler and Stenk, 1993).

If T5 α H is catalyzing a novel reaction to generate an unexpected product, then adoption of a different extraction and purification method may be necessary to detect this product. While the described hexane and SPE flow-through column was sufficient to detect taxa-4(5), 11(12)-diene, it may be insufficient to detect additional products. A single functional group modification can unexpectedly change the affinity of an alkane for a sorbent. For example, in biochemical studies of *in vitro* T5 α H activity, retention of the taxa-4(20),11(12)-dien-5 α -ol by the silica sorbent utilized in compound purification was found to be strongly dependent on the α or β orientation of the hydroxyl group that was ultimately determined by the T5 α H enzyme (Hefner et al., 1996).

Additionally, it is also possible that the product of T5 α H is not entirely soluble in the hexane utilized in this study for taxane extraction. Though T5 α H mediates the conjugation of a single hydroxyl group to C5 of the taxa-4(5), 11(12)-diene skeleton, this may be enough to affect its solubility in hexane, a point made more poignant if any product of T5 α H is only being produced in small quantities. For example, detection of 5(12)-oxa-3(11)-cyclotaxane in *N. sylvestris* utilized a pentane extraction, a solvent just a little more polar than hexane (Rontein et al., 2008). Additionally, a hexane:ethyl acetate (4:1 v/v) solution was utilized to extract taxa-4(20), 11(12)-dien-5 α -ol from the WAT11 yeast strain in the original T5 α H characterization paper (Jennewein et al., 2004). In both instances, the use of pentane or a hexane:ethyl acetate solution may have been required to fully solubilize the slightly more polar taxa-4(20), 11(12)-dien-5 α -ol or any other product that T5 α H may have produced. Thus, the inability to detect the detect taxa-4(20),11(12)-dien-5 α -ol or any other product of T5 α H may be due to the loss of the compound during extraction with hexane or purification with the SPE flow through column. In order to address this issue, a broad extraction for taxanes may be necessary. A broad method is described in Ketchum and Croteau (2006), where specialized solvents are utilized in the extraction of non-polar and polar taxanes. Following separate extraction processes utilizing solvents of different polarities, the extracts were combined, dried, concentrated, and analyzed by GC-MS (Ketchum, 2006). Additionally, the use of a slightly less non-polar solvent, such as pentane or a hexane:ethyl acetate solution, may be beneficial in detecting any product of T5 α H due to the increased solubility of its product in the extraction solvent.

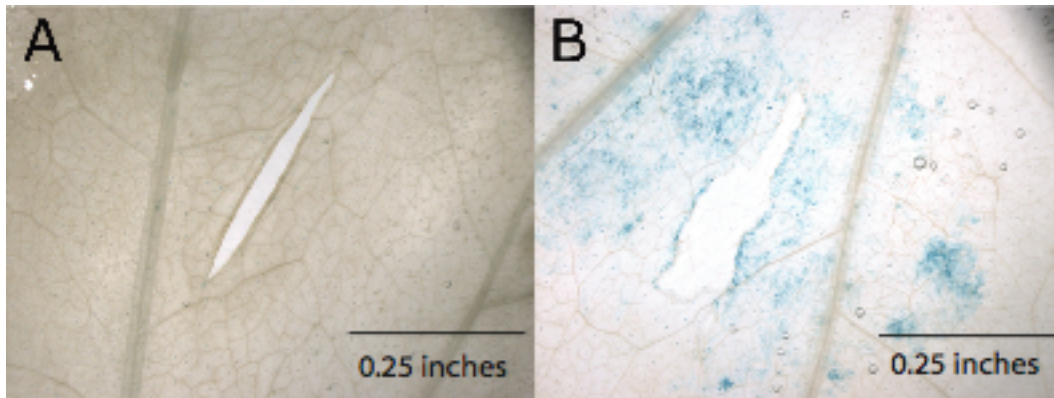


Figure 4.1: GUS activity in *Nicotiana benthamiana* following agroinfiltration

N. benthamiana leaf agroinfiltrated with *Agrobacterium tumefaciens* containing either A) no reporter construct, or B) pCAMBIA1301 containing 35S:intron-GUS following incubation in a histochemical GUS buffer at 37°C for 24 hours.

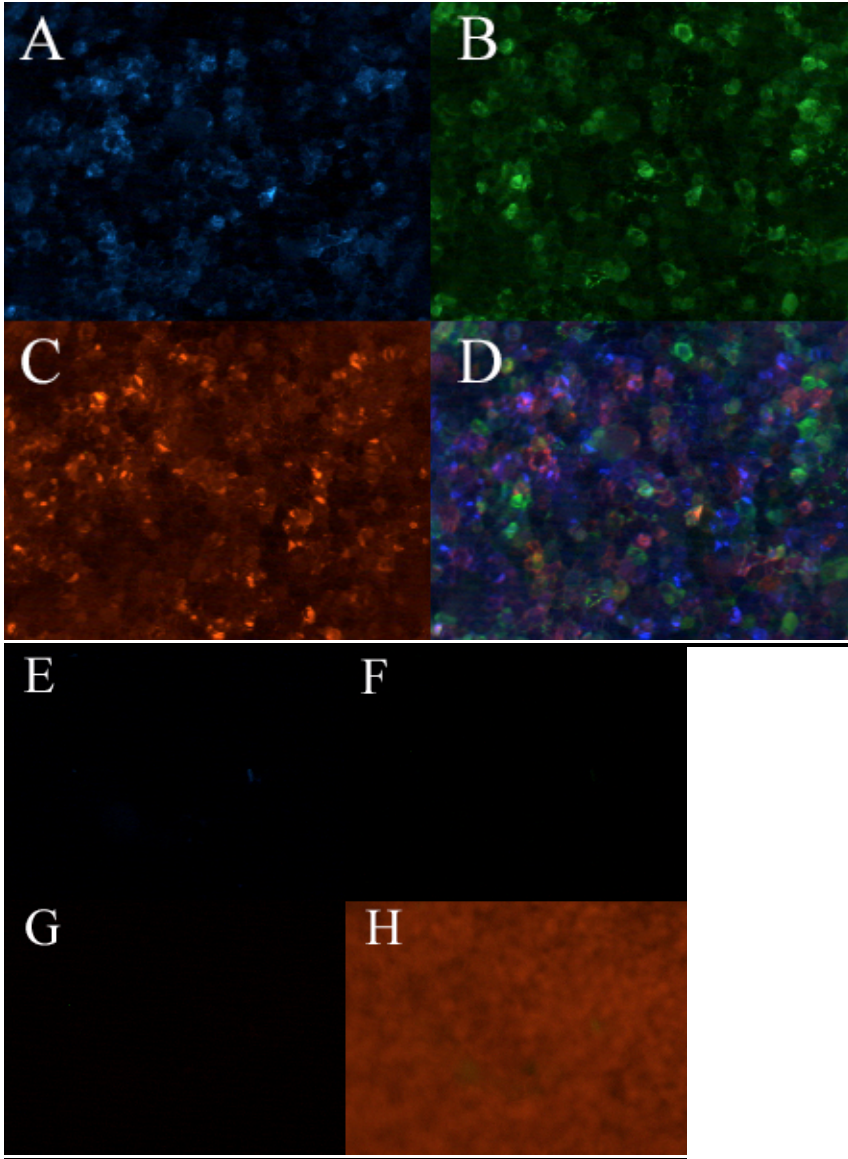


Figure 4.2: Demonstration of multiple transformation events following co-agroinfiltration with three different fluorescent reporter constructs

Fluorescent protein expression in a single *Nicotiana benthamiana* field of view as a result of *Agrobacterium* co-agroinfiltration. (A) represents 2x35S:ER-CFP fluorescence, (B) represents 2x35S:Plastid-YFP fluorescence, (C) represents 2x35S:PM-mCherry fluorescence, and (D) represents a merge of figures (A), (B), and (C) two days post agroinfiltration. All fluorescent proteins were driven by the double 35S promoter, and were agroinfiltrated into a single *N. benthamiana* in *Agrobacterium* strain C56C1. (E), (F), and (G) represent a single *N. benthamiana* field of view as a result of agroinfiltration with the pPZP221 empty vector. No autofluorescence is seen with the (E) CFP filter set, (F) YFP filter set, or (G) RFP filter set. Additionally, chlorophyll autofluorescence is observed with standard UV illumination in (H).

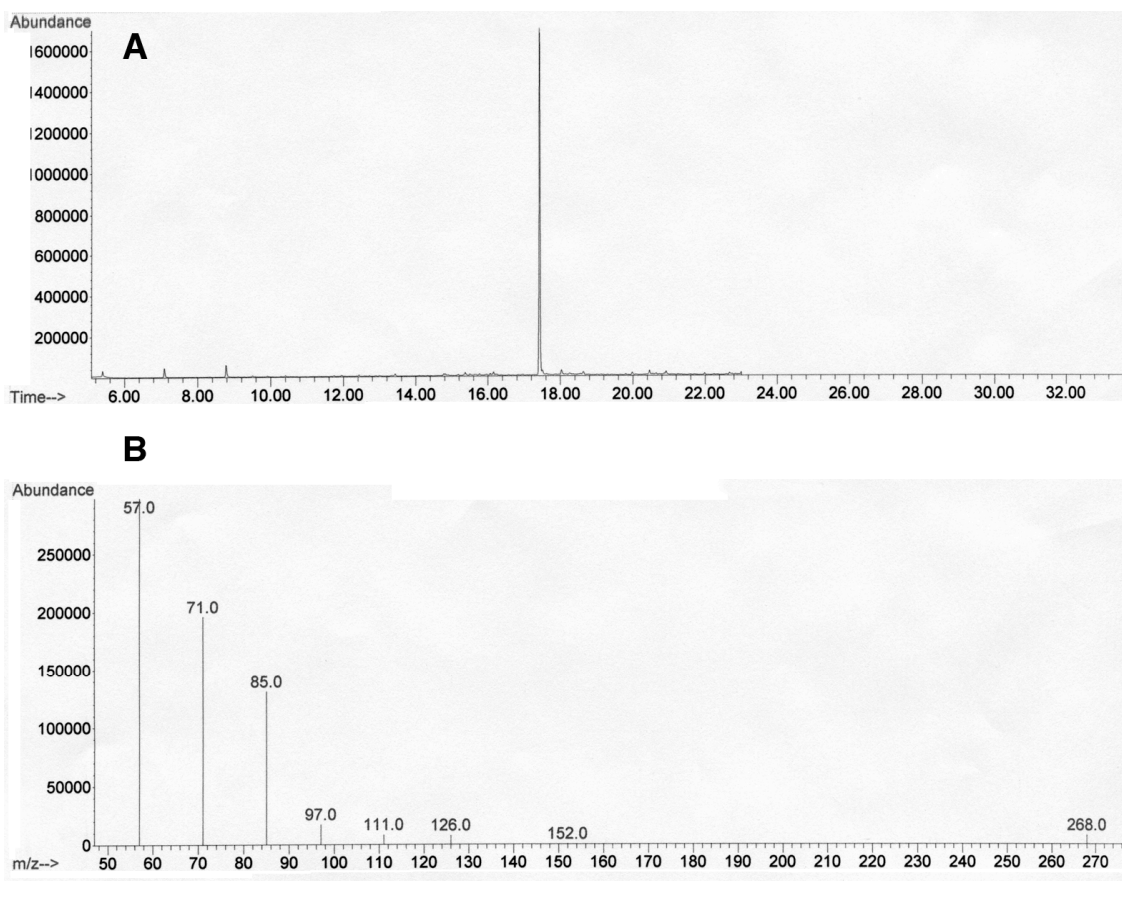


Figure 4.3: Total ion count and selected ion monitoring of nonadecane (10 pg/ μ l) after purification through an SPE silica column and GC-MS analysis

Major peak in the total ion count in (A) represents nonadecane eluting at approximately 17.5 minutes. Ion peaks in (B) represents fragment ions following ionization in MS analysis. These fragments conform to published fragmentation spectra of nonadecane.

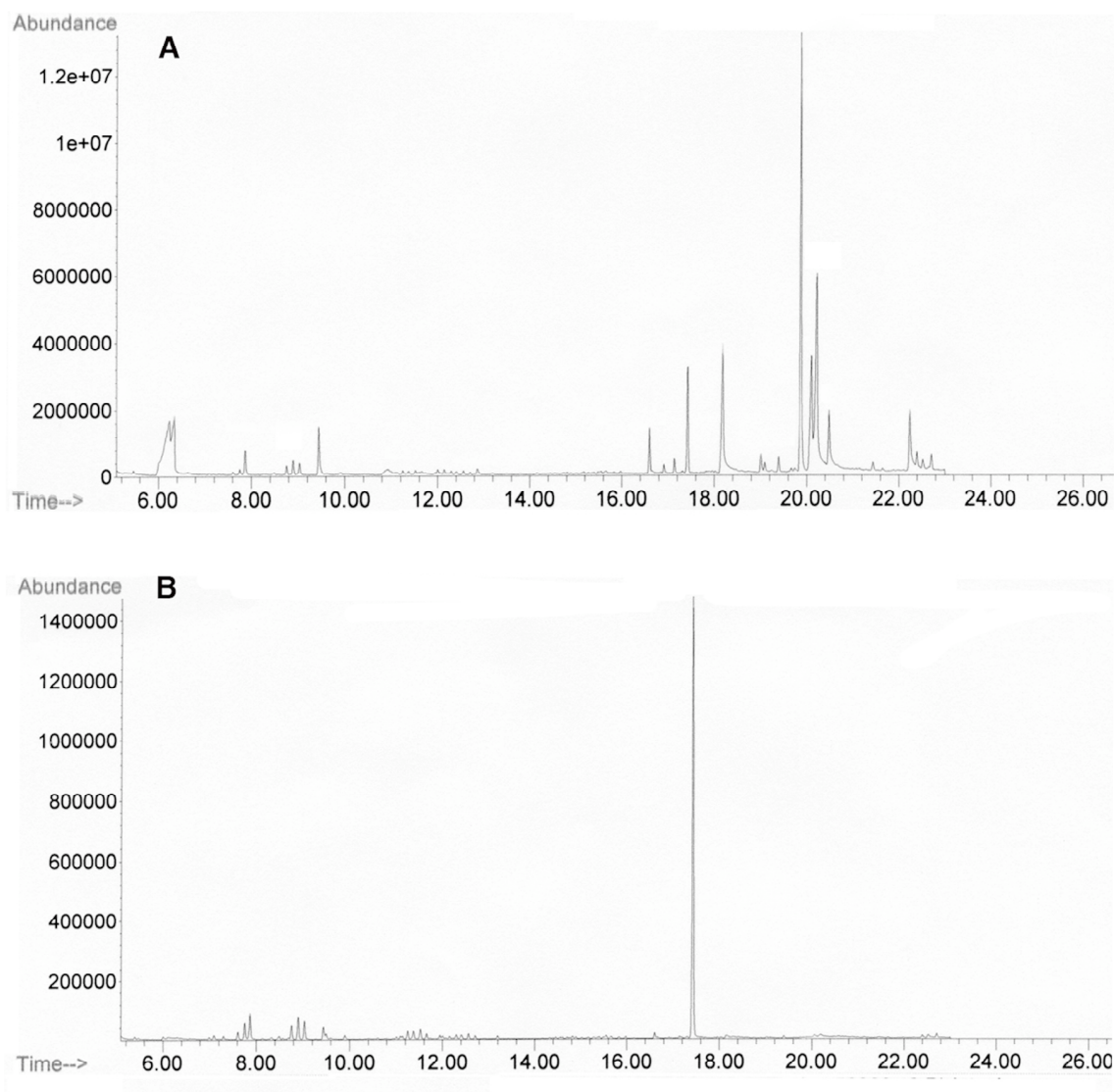


Figure 4.4: GC-MS comparison of *Nicotiana benthamiana* leaf tissue from non-SPE treated samples and SPE treated samples

(A) represents non-SPE treated samples, and (B) represents SPE-treated samples. Both samples represent 0.4 g of fresh weight collected five days post agroinfiltration and ground in liquid nitrogen and extracted in 1.5 ml hexane. Each sample was spiked with 10 pg/ μ l nonadecane, with nonadecane eluting at approximately 17.4 minutes as in Figure 4.1A.

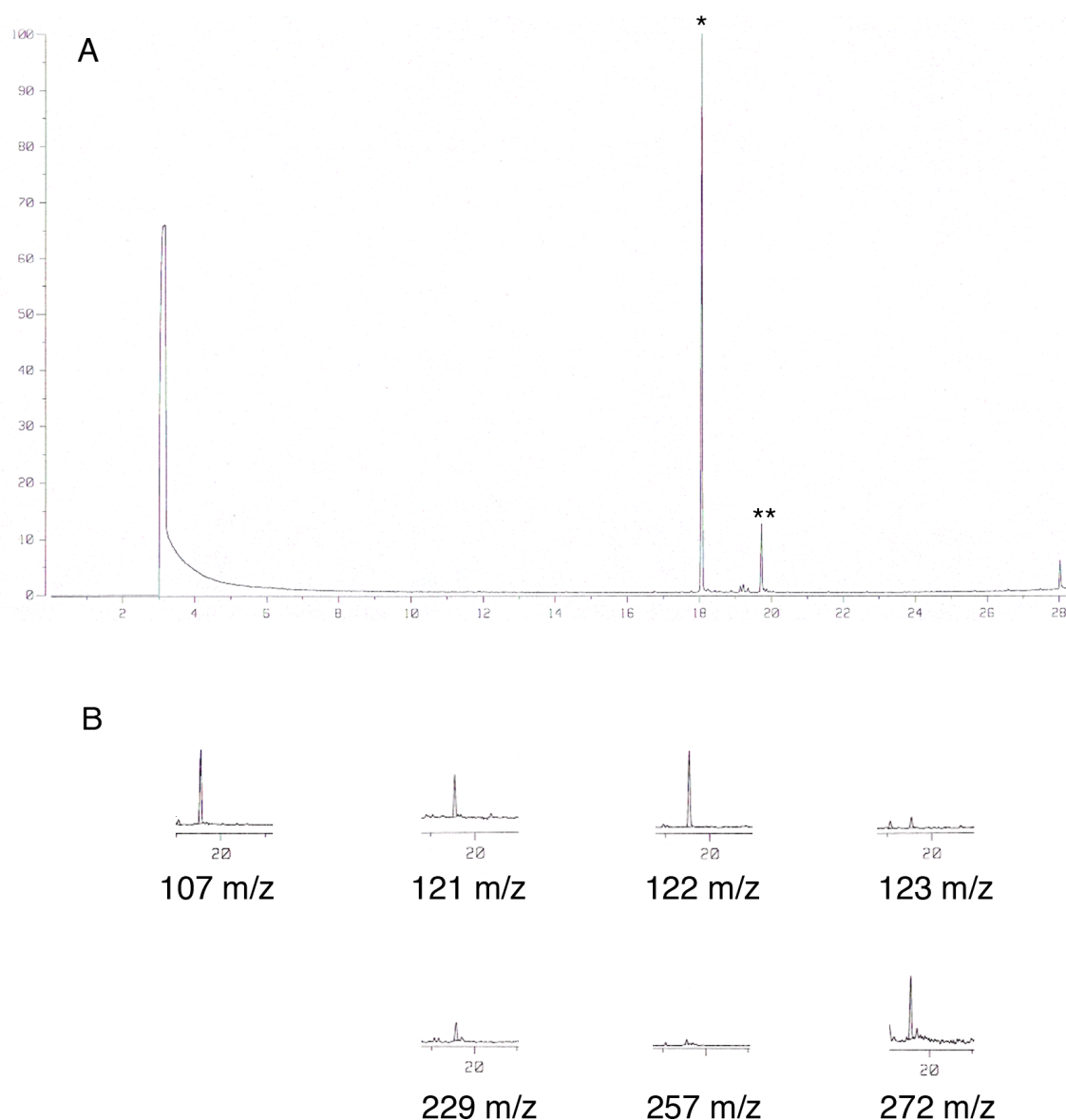


Figure 4.5: GC-MS analysis of taxa-4(5), 11(12)-diene following extraction of *Nicotiana benthamiana* agroinfiltrated with 35S:TASY

(A) represents total ion count, and (B) represents selective ion monitoring. Ground leaf fresh weight was 4 g, and was collected 5 days post agroinfiltration. In (A), * represents the internal standard, nonadecane with a retention time of 17.9 minutes, and ** represents taxa-4(5), 11(12)-diene with a retention time of 19.8 minutes. In (B), the fragment ions of taxa-4(5), 11(12)-diene are shown.

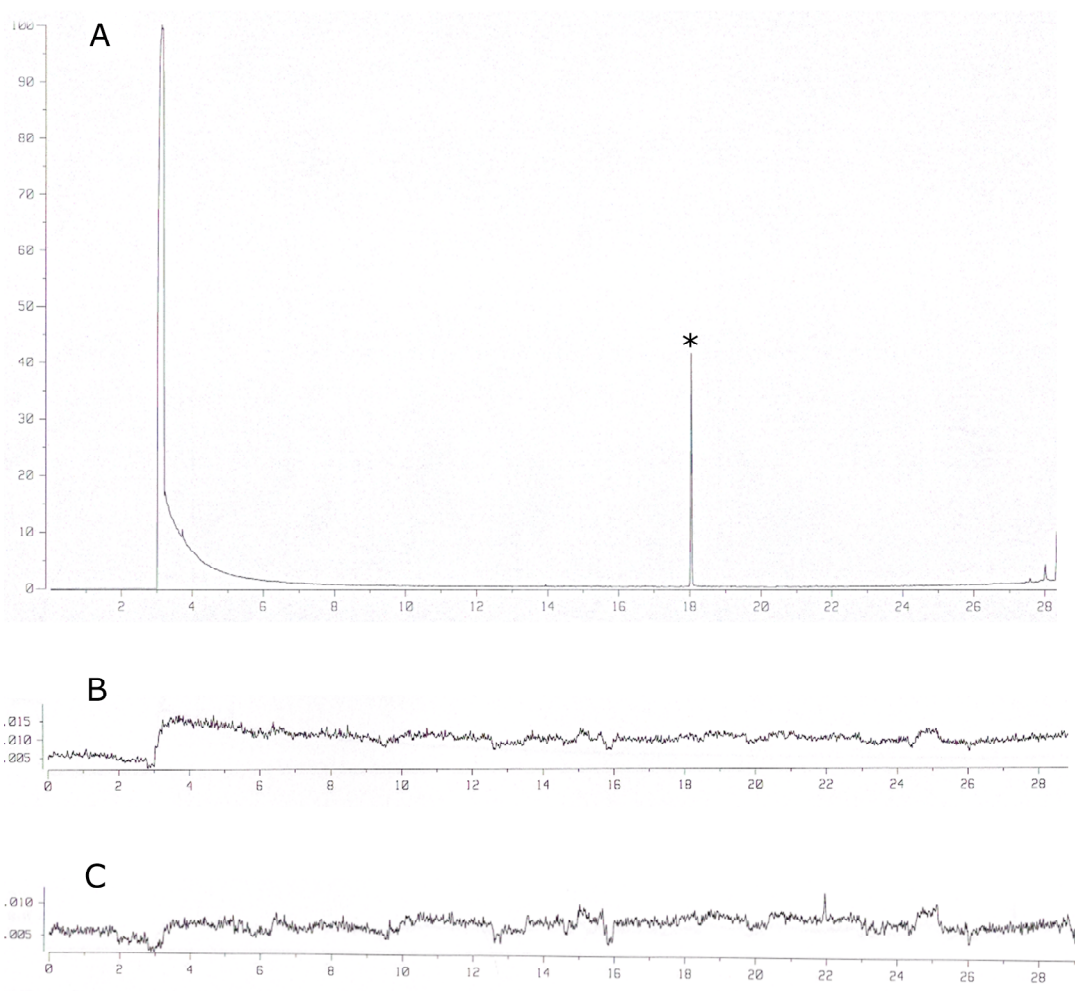


Figure 4.6: GC-MS analysis of *Nicotiana benthamiana* sample agroinfiltrated with 35S:T5αH

(A) represents total ion monitoring; (B) represents selected ion monitoring for the taxa-4(20), 11(12)-diene parent ion, 272 m/z; and (C) represents selected ion monitoring for the taxa-4(20), 11(12)-dien-5α-ol parent ion, 288 m/z. Ground leaf fresh weight was 4 g, and was collected 5 days post agroinfiltration. * represents the internal standard, nonadecane (10 pg/μl) with a retention time of 17.9 minutes. Neither taxa-4(5), 11(12)-diene or taxa-4(20), 11(12)-dien-5α-ol was detected.

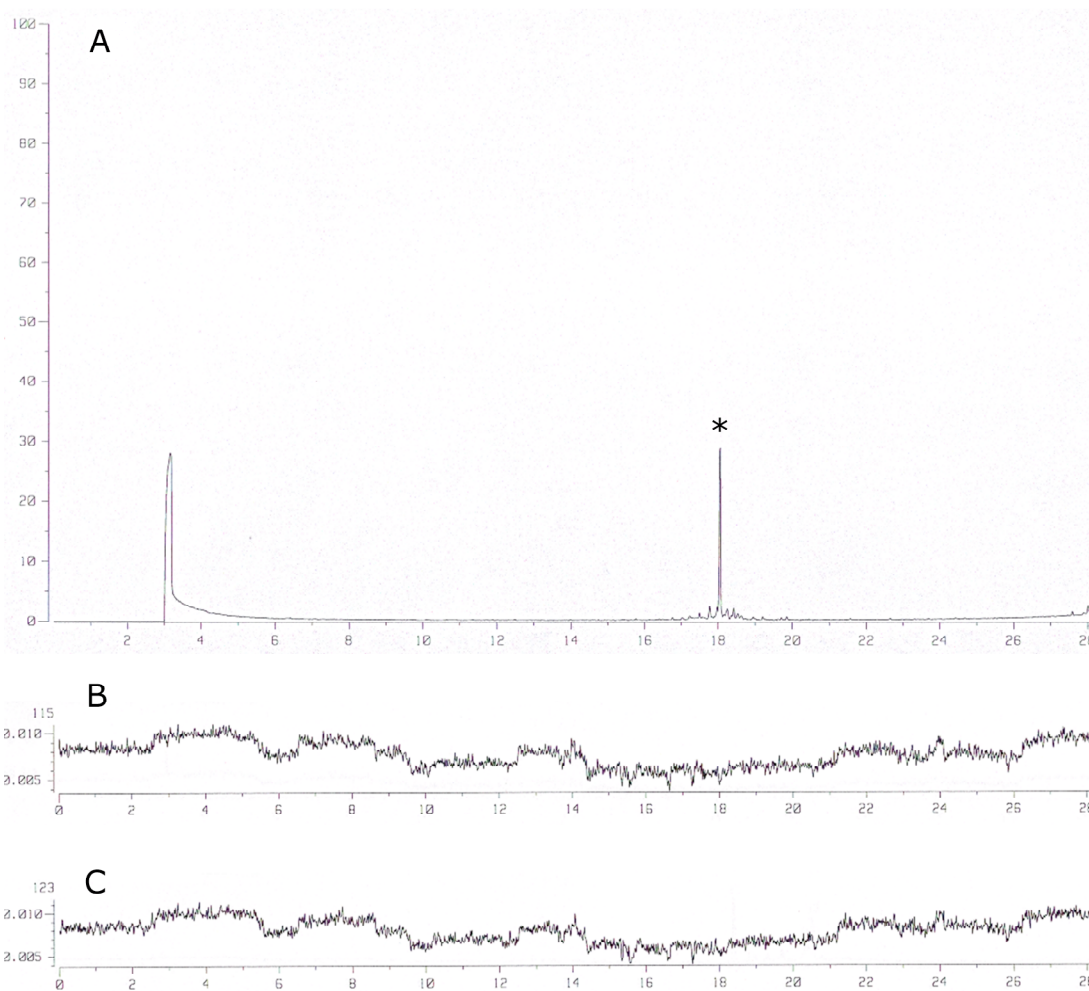


Figure 4.7: GC-MS analysis of *Nicotiana benthamiana* sample co- agroinfiltrated with 35S:TASY and 35S:T5 α H

(A) represents the total ion count monitoring; (B) represents selected ion monitoring for the taxa-4(20), 11(12)-diene parent ion, 272 m/z; and (C) represents selected ion monitoring for the taxa-4(20),11(12)-dien-5 α -ol parent ion, 288 m/z. Ground leaf fresh weight was 4 g, and was collected 5 days post agroinfiltration. * represents the internal standard, nonadecane (10 pg/ μ l) with a retention time of 17.9 minutes. Neither taxa-4(5), 11(12)-diene or taxa-4(20),11(12)-dien-5 α -ol was detected.

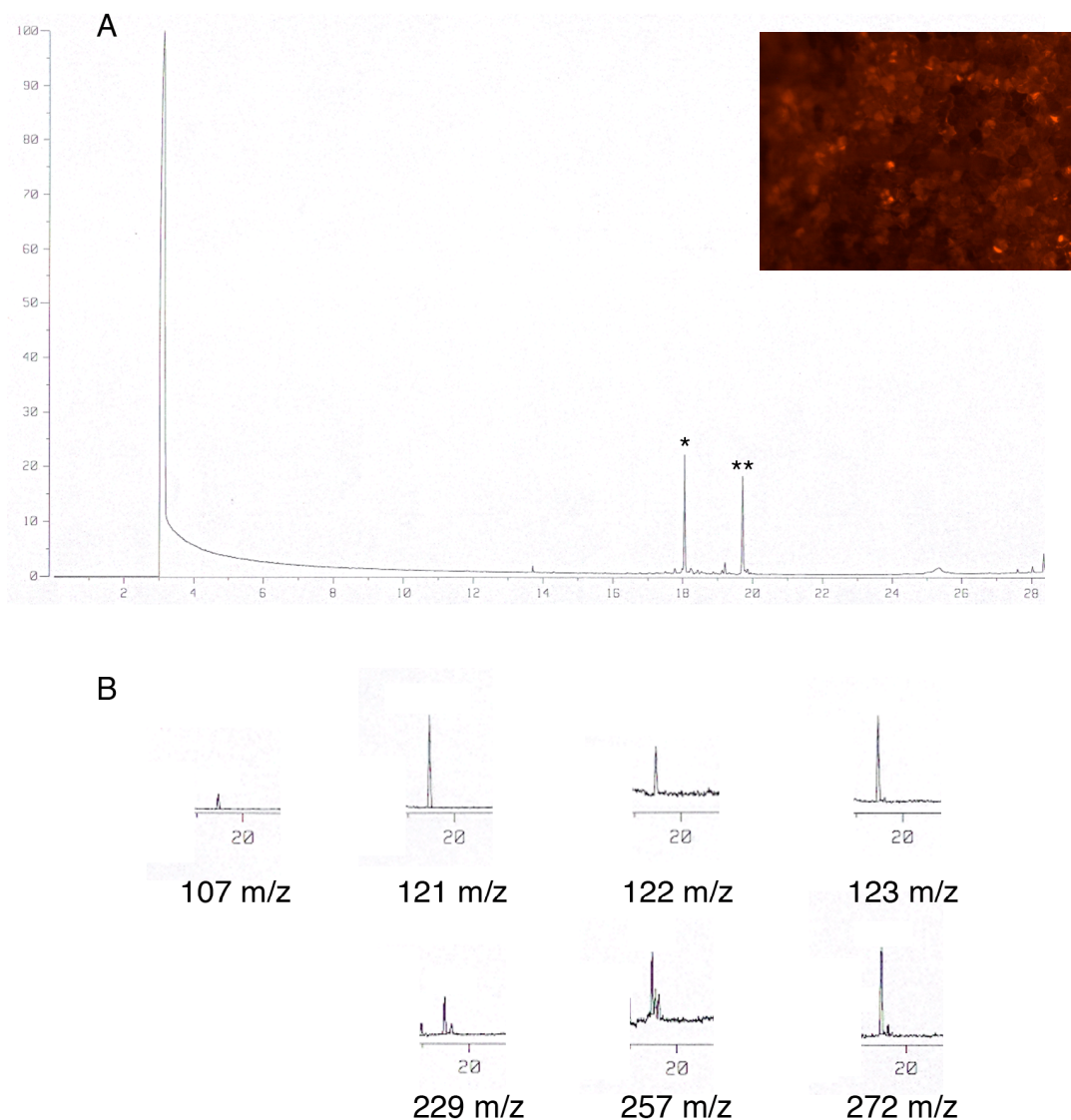


Figure 4.8: GC-MS analysis of *Nicotiana benthamiana* sample co-agroinfiltrated with TASY and the mCherry fluorescent protein (1:1)

(A) represents total ion monitoring, and (B) represents selective ion monitoring. Ground leaf fresh weight was 4 g, and was collected 5 days post agroinfiltration. In (A), * represents the internal standard, nonadecane with a retention time of 17.9 minutes, and ** represents taxa-4(5), 11(12)-diene with a retention time of 19.8 minutes. Inset shows mCherry fluorescence of leaf cells prior to tissue grinding in liquid nitrogen. *N. benthamiana* samples agroinfiltrated with 35S:TASY alone did not show any red autofluorescence (data not shown). In (B), the fragment ions of taxa-4(5), 11(12)-diene are shown.

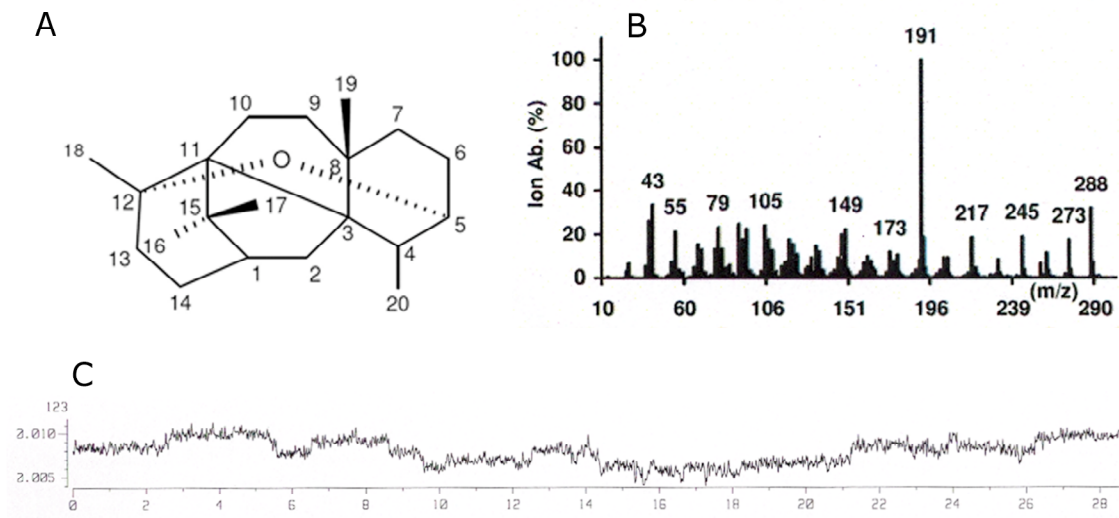


Figure 4.9: 5(12)-oxa-3(11)-cyclotaxane

(A) Structure for the 5(12)-oxa-3(11)-cyclotaxane detected in *Nicotiana sylvestris* by Rontein et al, 2008. (B) Mass spectrum for 5(12)-oxa-3(11)-cyclotaxane, as detected by Rontein et al, 2008. (C) SIM monitoring for the 288 m/z parent ion in the *N. benthamiana* samples examined in this study. No 288 m/z parent ion, and thus, 5(12)-oxa-3(11)-cyclotaxane was detected in the *N. benthamiana* sample.

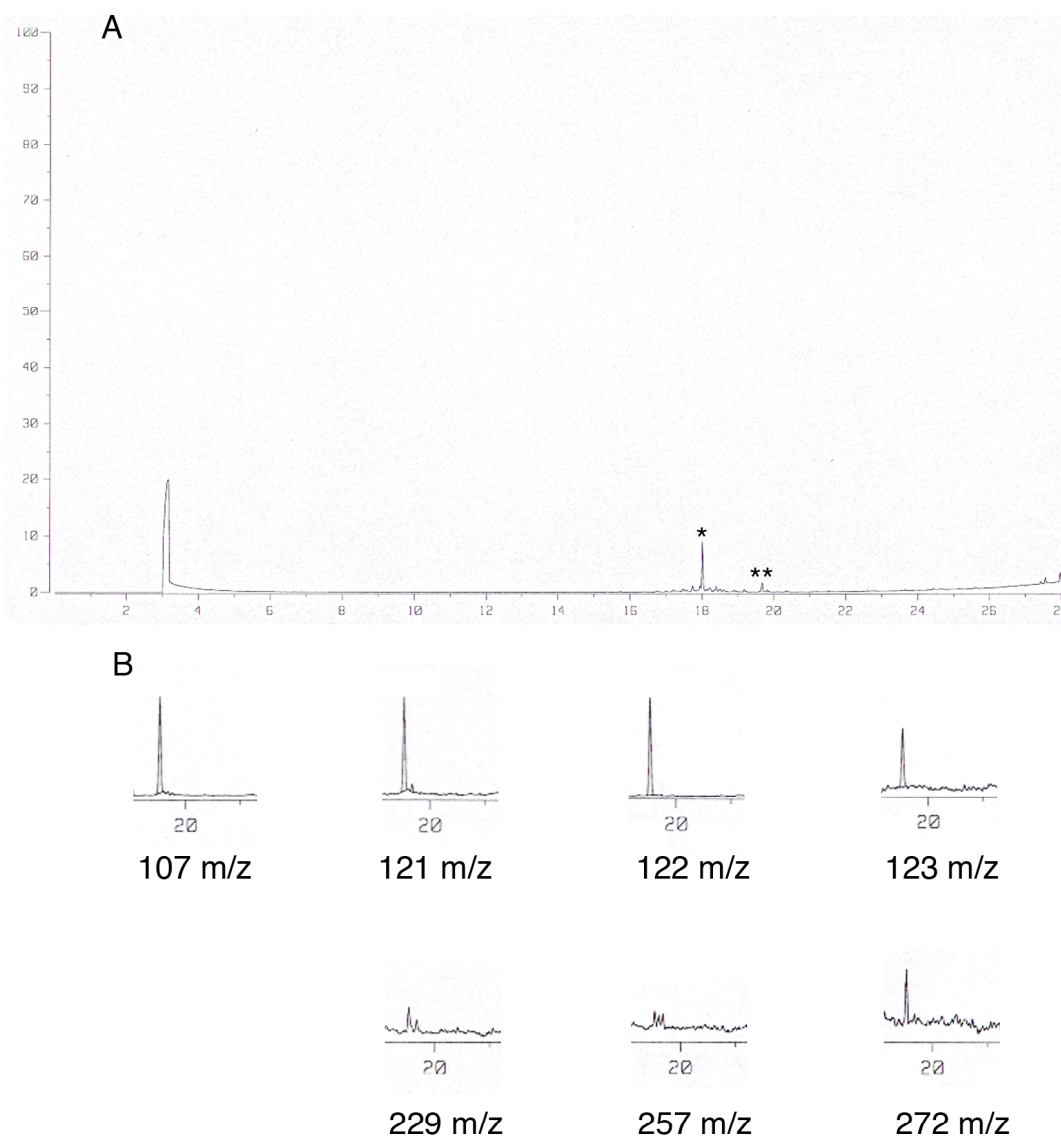


Figure 4.10: GC-MS analysis of *Nicotiana benthamiana* sample co-agroinfiltrated with TASY and T5 α H (9:1)

(A) represents total ion count monitoring, and (B) represents selective ion monitoring. Ground leaf fresh weight was 4 g, and was collected 5 days post agroinfiltration. In (A), * represents the internal standard, nonadecane with a retention time of 17.9 minutes, and ** represents taxa-4(5), 11(12)-diene with a retention time of 19.8 minutes.

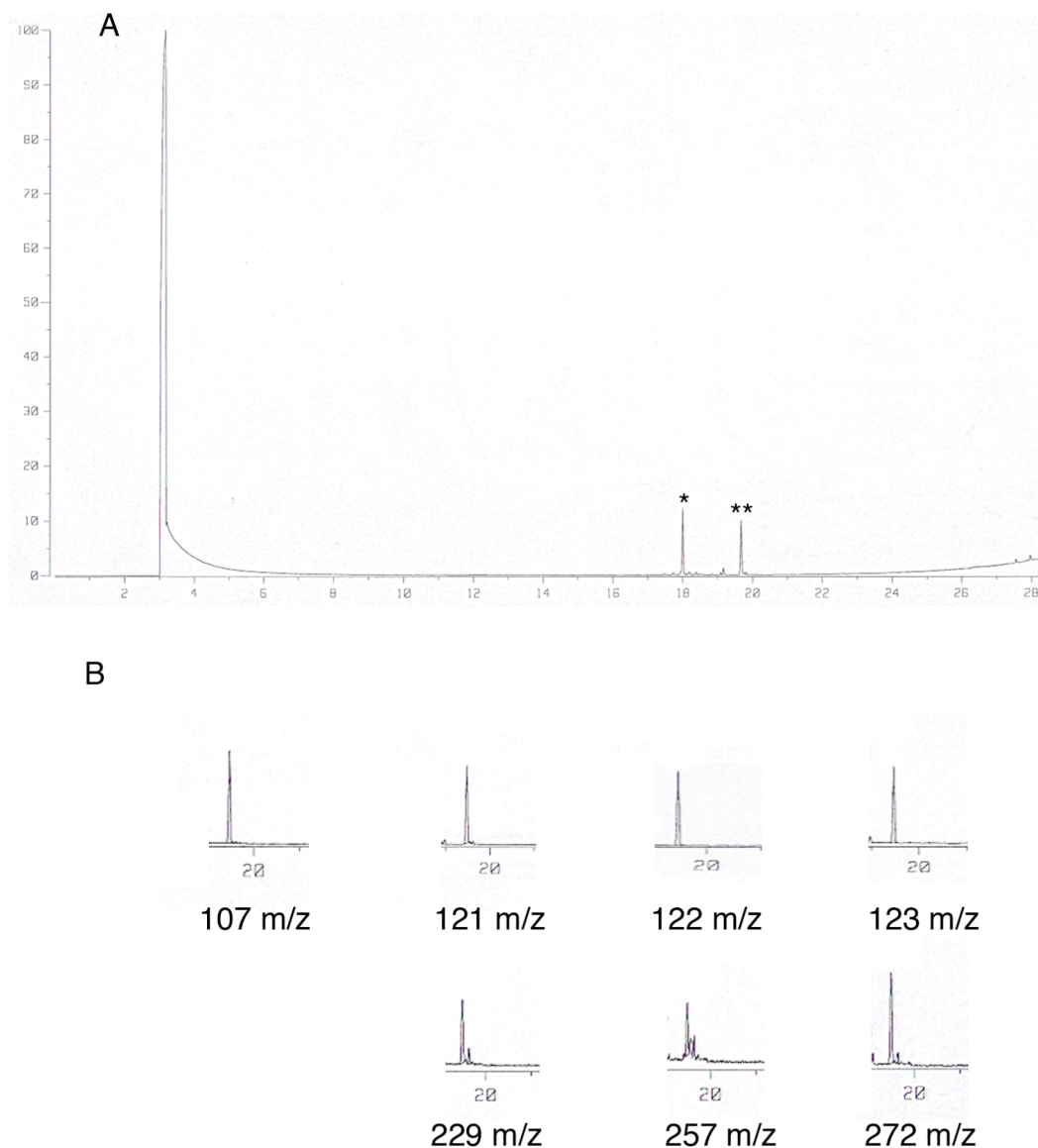


Figure 4.11: GC-MS analysis of *Nicotiana benthamiana* sample co-agroinfiltrated with TASY and the mCherry fluorescent protein (9:1)

(A) represents total ion count monitoring, and (B) represents selective ion monitoring. Ground leaf fresh weight was 4 g, and was collected 5 days post agroinfiltration. In (A), * represents the internal standard, nonadecane with a retention time of 17.9 minutes, and ** represents taxa-4(5), 11(12)-diene with a retention time of 19.8 minutes.

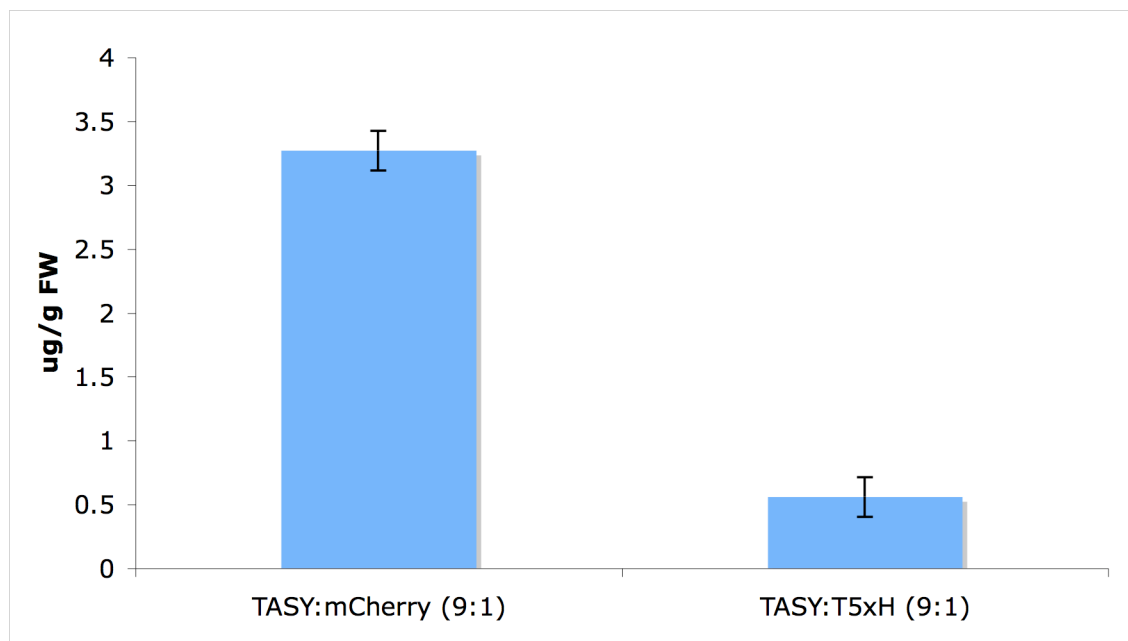


Figure 4.12: Quantification of taxa-4(5), 11(12)-diene following co-agroinfiltration of *Nicotiana benthamiana* with either 35S:TASY and 35S:mCherry or 35S:TASY and 35S:T5 α H

Both co-agroinfiltrations were performed at a 9:1 ratio, respectively. 4 g samples were collected 5 days post co-agroinfiltration and purified through a solid phase extraction flow-through column.

CHAPTER 5

DEVELOPMENT OF AN *AGROBACTERIUM*-MEDIATED TRANSFORMATION PROTOCOL FOR *TAXUS* CELLS IN CULTURE

5.1 Introduction

5.1.1 Variability in *Taxus* Cell Culture

One of the major limitations in any plant cell culture system is variable secondary metabolite yield (Roberts, 2007; Roberts and Shuler, 1997). *Taxus* cell culture is no different, with concentrations ranging from 0 mg/L to upwards of 80 mg/L and higher (Choi et al., 2002; Naill and Roberts, 2005). Different approaches have been used to stabilize and increase paclitaxel production in *Taxus* cell culture. Traditional methods have included environmental optimization and elicitation (Tabata, 2004; Yukimune et al., 1996), while novel methods have focused on flow cytometric analysis and application of bioreactor technology (Kolewe et al., 2010; Kolewe et al., 2008). While these methods have provided valuable information for the increased production of paclitaxel, they do not directly address the biological impediments of variable paclitaxel yield in cell culture. However, utilizing advances in plant molecular biology can not only elucidate the mechanisms of metabolite variability on a single cell or whole culture level, but also provide avenues for the targeted metabolic engineering of *Taxus* cells for the generation of superior paclitaxel-accumulating cell lines.

5.1.2 *Agrobacterium*-mediated Transformation

There are numerous methods of transgene delivery available to plant scientists. Polyethylene glycol (PEG), electroporation, and biolistics have been used in various plant

systems for the generation of stable transformants (Herrera-Estrella et al., 2005; Nehra et al., 2005; Rakoczy-Trojanowska, 2002). While each of these approaches has been used with varying success, *Agrobacterium tumefaciens* offers the most elegant tool for integration of non-native DNA into a plant genome.

Evolution has conferred *A. tumefaciens* with native protein machinery that transfers and integrates a portion of its genome (T-DNA) into a plant host genome. This T-DNA is defined by T-DNA Left and Right Borders, and is transferred and integrated into the plant host genome by native *Agrobacterium* proteins encoded by the *vir* genes (Gelvin, 2003a). Upregulation of these *vir* genes, such as induction by the bacterial hormone acetosyringone, is central to the success of *Agrobacterium*-mediated stable transformation of plants (Gelvin, 2000).

In addition, *Agrobacterium* often yields single integration events. This permits *Agrobacterium*-mediated transformation to avoid complex insertion patterns, such as inverted tandem repeats, associated with biolistics, PEG-mediated transformation, and electroporation. However, the success of *Agrobacterium*-mediated transformation is highly dependent on the host used; while numerous angiosperms are amenable to *Agrobacterium*-mediated transformation, gymnosperms have proven far more challenging. The mechanisms dictating the range of host/pathogen interaction is not known with any certainty, though it is speculated that interaction between specific *Agrobacterium* virulence genes/genetic background and the plant host plays a role in determining pathogenicity. For example, when the Ti plasmid pTiBo542 and the virulence genes contained within it are utilized within its native strain, *Agrobacterium* strain Bo542, directed transformation of legumes is limited (Gelvin, 2003a). However,

when pTiBo542 is transformed into *Agrobacterium* strain C58, transformation efficiency of soybean is significantly increased (Hood et al., 1987). Additionally, specific *Agrobacterium* virulence factors may contribute to potential transformation efficiency. Constitutive expression of *virG* in *Agrobacterium* strain LBA4404 confers a hyper-virulent phenotype, as demonstrated by the near 100% transformation efficiency of *Catharanthus roseus* cell culture system following a liquid-liquid co-cultivation (van der Fits and Memelink, 2000). It is prudent to note, however, that expression levels of *vir* factors alone may not be sufficient to determine infectivity; in many cases, the plant host strain plays a significant role in determining the outcome of *Agrobacterium* co-cultivation (Gelvin, 2003b; Gelvin, 2006). This is best demonstrated by the continual use of specific plant lines for transformation during transgenic crop development in the agricultural biotechnology sector.

5.1.3 Targeted Metabolic Engineering of *Taxus*

The targeted metabolic engineering of *Taxus* requires two factors to be addressed before it can be effectively implemented. First, molecular targets must be identified that regulate aspects of paclitaxel metabolism. Second, a reliable method must be developed for the stable introduction of transgenes into *Taxus*. Previous work has identified transcriptional bottlenecks in paclitaxel biosynthesis (Nims et al., 2006), as well as a putative methyl jasmonate-responsive transcriptional regulator of paclitaxel biosynthesis (Nims et al., unpublished data). The goal of this work was to develop a reliable transformation protocol for the introduction of transgenes into *Taxus* cells in culture.

5.2 Materials and Methods

A complete description of the materials and methods is located in Chapter 2. Of particular interest is *Taxus* cell culture (2.2), fresh weight measurement (2.2.3), binary plant expression vectors (2.4.3), and *Agrobacterium*-mediated transformation (2.5.1).

5.3 Results

5.3.1 Examination of the Effect of Cefotaxime in *Taxus* Cells in Culture

Following *Agrobacterium* transformation, use of a bactericide is often utilized to eliminate *Agrobacterium* population levels during the plant recovery and selection period. Effective use of an appropriate bactericide ensures that residual *Agrobacterium* from the co-cultivation does not out-compete and overwhelm sensitive plant cells during the recovery and selection process. Cefotaxime is routinely utilized as a bactericide following *Agrobacterium* transformation, as it only affects bacterial cell wall synthesis and not plant cell wall synthesis. Cefotaxime, a β -lactam antibiotic, inhibits the final transpeptidation step of peptidoglycan synthesis in the cell walls in a variety of gram-negative and gram-positive bacteria, eventually inducing cell death (LeFrock, 1983).

Addition of 150 or 300 $\mu\text{g/ml}$ cefotaxime did not negatively impact growth and viability of *Taxus* cell lines P991, P93AF, PO93X, or CO93D (eight to nine days post subculture) (Figure 5.1). Interestingly, the addition of cefotaxime appeared to stimulate P991 and CO93D growth during the incubation period of 28 days (Figure 5.1A and Figure 5.1B). However, increasing cefotaxime concentrations in both P991 and CO93D were not correlated with gains in fresh weight.

The lack of a negative impact of 300 µg/ml cefotaxime on growth and viability of *Taxus* cell lines P93AF and PO93X, combined with the positive impact on growth in *Taxus* cell lines P991 and CO93D, suggests that 300 µg/ml cefotaxime could be beneficial following *Agrobacterium* co-cultivation.

5.3.2 Examination of Hygromycin B Selection in *Taxus* Cells in Culture

Hygromycin B is an aminoglycosidic antibiotic that inhibits protein synthesis by disrupting translocation and promoting mistranslation at the 30S ribosome subunit formation (Figure 5.2A) (Gritz and Davies, 1983; McGaha and Champney, 2007). *Taxus* cell lines P93AF, PO93X, and CO93D (eight to nine days post subculture) were tested against hygromycin B at 0, 50, and 100 µg/ml concentrations on solid B5 media supplemented with 300 µg/ml cefotaxime. Growth was inhibited at each hygromycin B concentration in all *Taxus* cell lines relative to their respective control lines, with cell death occurring even on the lowest hygromycin B concentrations as early as two weeks post-plating (Figure 5.3). Due to the presence of autofluorescent stress compounds produced by *Taxus*, viability could not be determined by a FDA viability assay. However, each hygromycin treatment also utilized a group of plated *Taxus* cells that were not weighed, but left at room temperature in the dark. These groups of *Taxus* cells did not show any growth beyond the measured time points, verifying that *Taxus* growth was arrested (data not shown). The range of effective hygromycin B concentrations that were inhibitory towards *Taxus* cell growth is similar to hygromycin B concentrations used in other plant systems in the literature (Alves et al., 2009; Cseke et al., 2007; Ishizaki et al., 2008; Lu et al., 2007; Rajam and Kumar, 2006). Thus, hygromycin B may serve as a potential selectable marker in the *Taxus* system, though this ultimately will have to be

evaluated across all *Taxus* cell lines following an established stable transformation protocol to determine efficacy.

5.3.3 Examination of Glufosinate Ammonium Selection in *Taxus* Cells in Culture

The chemical structure of glufosinate ammonium consists of a glutamic acid analogue moiety (phosphinothricin) and two alanine residues (Figure 5.2B). Effect of the herbicide occurs only after native intracellular peptidases remove the alanine residues, allowing the phosphinothricin to act a competitive inhibitor of glutamine synthetase. As a result, toxic levels of ammonia accumulate in the cell, eventually killing it. Resistance to glufosinate ammonium is conferred by the *bar* gene, originally isolated from *Streptomyces hygroscopicus* (Thompson et al., 1987). The *bar* gene acetylates the amino groups in glufosinate ammonium, disallowing the necessary peptidase hydrolysis required for competitive inhibition.

Taxus cell lines P991, P93AF, PO93X, and CO93D (eight to nine dayspost subculture) were tested against glufosinate ammonium at 0, 20, 40, 200, and 400 µg/ml concentrations in solid B5 media supplemented with 300 µg/ml cefotaxime. P991 growth was inhibited at glufosinate ammonium concentrations as low as 20 µg/ml, with cell death occurring as early as two weeks post-plating (Figure 5.4A). P93AF growth was not inhibited at the lower glufosinate ammonium concentrations, with cell death only occurring at 200 and 400 µg/ml of glufosinate ammonium (Figure 5.4B). PO93X growth was not inhibited even at the highest glufosinate concentrations (Figure 5.4C). The trend of growth inhibition in CO93D was similar to P93AF, with only higher glufosinate ammonium concentrations (200 and 400 µg/ml) inhibiting growth and causing cell death (Figure 5.4D).

Based on these results, it appears that glufosinate is not a suitable selection agent by which to select for positive *Taxus* transformants, as the survivability of *Taxus* cell lines P93AF and PO93X on ≤ 200 $\mu\text{g/ml}$ of glufosinate ammonium exceeds the concentrations that are normally used in selection of plant species such as *A. thaliana*, tobacco, and pine (Iamtham and Day, 2000; Krolikowski et al., 2003; Nigro et al., 2008).

5.3.4 Examination of Traditional *Agrobacterium*-mediated Transformation

Transformation of *in vitro* suspension plant material is usually achieved through use of a plant suspension/*Agrobacterium* co-cultivation (Figure 5.5) (Tzfira and Citovsky, 2006; van der Fits et al., 2000; Wenck et al., 1999). In general, *Agrobacterium* is grown in a large-scale culture with nutrient-rich media and acetosyringone, pelleted, resuspended in plant growth media, and co-cultivated with the plant suspension of choice. The parameters varied in this study of traditional liquid-liquid co-cultivation included the reporter construct used (35S:GFP, 35S:GUS, and 35S:intron-GUS), plant cell line (P991, P93AF, PO93X, and CO93D), *Agrobacterium* strain (GV3101, LBA4404, and C56C1), acetosyringone concentration (0, 10, 100 μM), duration of co-cultivation (1-2 days), and duration of recovery period on *Taxus* growth media supplemented with 300 $\mu\text{g/ml}$ cefotaxime prior to plating on selection media (1, 3, and 7 days). These parameters are summarized in Table 5.2.

Additionally, following the publication of an *Agrobacterium*-mediated transformation protocol (Ketchum et al., 2007), reproduction of the described method was attempted utilizing *Agrobacterium* strain EHA105 (carrying pCAMBIA1391 containing 35S:intron-GUS) and sonication of the *Taxus* cells (Table 5.3). Otherwise, the protocol was repeated as described in Ketchum et al. (2007).

Initial work indicated that *Taxus* green autofluorescence of the cell wall occurred in all *Taxus* cell lines following stress of the culture (Figure 3.1A). Due to this green autofluorescence, GFP (green fluorescent protein) was precluded as a reporter choice because the overlap of cell wall autofluorescence and GFP emission spectra. Therefore, 35S:GUS and 35S:intron-GUS was chosen as reporter genes to evaluate the applicability of traditional *A. tumefaciens* co-cultivation for gene transfer to *Taxus* cell lines *in vitro*. 35S:intron-GUS was verified as functional in *Taxus* in the literature, confirming that the intron does not interfere with GUS activity in *Taxus* (Ketchum et al., 2007).

Regardless of the parameters manipulated, *Agrobacterium*-mediated transformation of *Taxus* was unsuccessful utilizing either traditional liquid-liquid co-cultivation or the described protocol by Ketchum et al. (2007). No reporter expression was ever observed, and cell death followed co-cultivation with *Agrobacterium* at all parameter combinations (Table 5.2 and Table 5.3). The cell death associated with *Agrobacterium* co-cultivation period is not uncommon in plant cell culture systems and is indicative of unsuccessful cell-to-cell attachment between pathogen and host or a failure to transfer/integrate the T-DNA (Gelvin, 2005; Gelvin, 2006). While speculative, it seems likely that T-DNA transfer did not occur from pathogen to host; previous particle bombardment work has demonstrated that a variety of reporter genes and promoters are functional in *Taxus* shortly following gene transfer (chapter 3). Thus, the stress resulting from unsuccessful cell-to-cell attachment between *Agrobacterium* and a plant host would likely induce a stress response from the plant cells, eventually leading to cell death, as was observed in *Taxus* (S. Gelvin, personal communication).

5.3.5 Examination of a Novel Method of *Agrobacterium*-mediated Transformation

While plant biologists have long used *Agrobacterium* as a delivery system for transgenes into plants, relatively few have studied the mechanism of T-DNA transmission into the host. Data from microbiologists suggest that many of the conditions commonly used to grow *Agrobacterium* in large-scale culture for plant transformation inhibit expression of *Agrobacterium* virulence genes necessary for transfection and integration of transgenes into a plant host (Gelvin, 2006). These data suggest that traditional transformation protocols used in the generation of transgenic plant species are merely sufficient for transfection in those plant systems and may not be optimized.

A modified protocol used for *Agrobacterium vir* gene induction was utilized to prime *A. tumefaciens* prior to co-cultivation with *Taxus*. The only parameters that were manipulated included *Taxus* cell line (P991, P93AF, PO93X, and CO93D), *Agrobacterium* strain (GV3101, LBA4404, C56C1, and EHA105), and the reporter construct (35S:intron-GUS or 35S:mcherry) (Table 5.4). *A. tumefaciens* was grown in small-scale culture in YEP media with the necessary antibiotics overnight, transferred to a minimal nutrient media with the necessary antibiotics for large-scale culture, and finally transferred to *vir* gene induction media supplemented with 100 μ M acetosyringone overnight before co-cultivation with *Taxus* (Chapter 2.5.1 and Figure 5.6) (Gelvin, 2006).

Temporally separating *A. tumefaciens* vegetative growth and *vir* gene induction did not yield any mCherry fluorescence or intron-GUS activity in any *Taxus* cell line. Overall morphology of the *Taxus* cells was better following co-cultivation, with some aggregates displaying the characteristic pale cream color indicative of cell health (data not shown).

5.3.6 Dry Co-Cultivation of *Agrobacterium* and *Taxus* Cells in Culture

Previous transformation attempts with *Taxus* and *Agrobacterium* utilized a liquid/liquid co-cultivation system due to initial growth of both organisms in suspension and ease of experimental set up. This liquid/liquid co-cultivation system did not yield any positive results as described above. However, other co-cultivation systems are utilized in the literature for the generation of transgenic plant lines.

Dry co-cultivation is often used in recalcitrant plant systems for the production of stable transgenic lines, such as *Zea mays*, *Medicago sativa*, and *Brachypodium distachyon* (Frame et al., 2006; Montague et al., 2007; Vogel and Hill, 2008). Intact plant tissue is often used in these transformation systems, either as direct material for transformation, or for the generation of callus tissue for transformation. Though the exact methods for these transformation systems vary, similar steps include the priming of *Agrobacterium* prior to the solid co-cultivation, application of a small volume of *Agrobacterium* directly to the plant material meant for transformation in a Petri plate and co-cultivation in the absence of solid media for a couple of days, and a recovery period of approximately several days on solid plant growth media supplemented with cefotaxime.

Successful transformation of *Taxus* was achieved utilizing this dry transformation method (described in chapter 2.5.1), where only *Taxus* cell line (P991, P93AF, PO93X, and CO93D) and *Agrobacterium* strain were varied (GV3101, LBA4404, EHA105, C56C1) (Table 5.5). β -Glucuronidase activity was observed in *Taxus* cell line PO93X utilizing *Agrobacterium* strain EHA105 (carrying 35S:intron GUS within pCAMBIA1301) following a two-day co-cultivation period and a two-day recovery

period on solid Gamborg B5 media (Figure 5.7). Interestingly, *Taxus* cell line PO93X was also one of the lines successfully transformed by Ketchum *et al.* (2007). This overlap of successful *Taxus* and *Agrobacterium* genotypes suggests that a potentially unknown genetic factor is partially responsible for the amenability of this cell line for the successful transfer of T-DNA from pathogen to plant host.

Selection of putative transformants on Gamborg B5 supplemented with hygromycin B did not yield growth of positive transformants. This is likely related to the low transformation efficiency and the subsequently caused island effect, where viable positive transformants are isolated from other viable positive transformants by numerous dying or necrotic negative transformants during selection. *Taxus* cells themselves are not robust like other plant *in vitro* systems, explaining the lack of growth when conditions are not ideal for mitotic cellular division.

Parameters have not yet been optimized for this solid co-cultivation method. Transformation efficiencies are well under 1%; while this efficiency is no worse than many other reported *Agrobacterium*-mediated transformation efficiencies, higher transformation efficiencies do increase the likelihood of selecting for positive transformants during the regrowth stage.

5.4 Discussion and Conclusions

5.4.1 Selection of *Taxus* Transformants

Taxus cell lines (P991, P93AF, PO93X, and CO93D) were examined for toxicity against the selective agents cefotaxime, hygromycin B, and glufosinate. *Taxus* was not negatively affected by the bactericide, cefotaxime, at any concentration (Figure 5.1).

Each *Taxus* cell line was sensitive to hygromycin B at all tested concentrations (Figure 5.3), while *Taxus* sensitivity to common glufosinate concentrations utilized in the literature was restricted to *Taxus* cell line P991 (Figure 5.4).

The response of *Taxus* cell lines P93AF and PO93X does not represent a departure from the utilization of cefotaxime as a bactericide, in that it does not impact growth or viability of a plant cell culture system (Danilova and Dolgikh, 2004; Humara and Ordás, 1999; Ling et al., 1998). In contrast, the stimulation of cell division and growth in *Taxus* cell lines P991 and CO93D represents a phenomenon that has previously been observed in other plant callus culture systems, including *Zea mays*, *Passiflora edulis*, *Triticum aestivum*, and *Hordeum vulgare* (Bhau and Wakhlu, 2001; d'Utra Vaz et al., 1993; Mathias and Boyd, 1986; Mathias and Mukasa, 1987). While specific interactions appear to be genotype specific in these systems, similar to the response in the *Taxus* system, there is no validated mechanism describing the stimulation of cell division and growth by cefotaxime in plant cell culture. However, it is speculated that breakdown products from cefotaxime *in vitro* may possess auxin-like activity (Danilova and Dolgikh, 2004; Mathias and Mukasa, 1987). While the mechanism of cefotaxime-mediated growth stimulation in *Taxus* cell lines P991 and CO93D is unresolved, it has been demonstrated that 150 or 300 µg/ml cefotaxime does not negatively impact viability and growth in any *Taxus* cell line.

Taxus cell lines P991, P93AF, PO93X, and CO93D are uniformly sensitive to commonly utilized concentrations of hygromycin B (Alves et al., 2009; Cseke et al., 2007; Ishizaki et al., 2008; Lu et al., 2007; Rajam and Kumar, 2006). However, the *Taxus* cell lines examined are not uniformly sensitive to the herbicide glufosinate, despite

P991, P93AF, and PO93X being derived from the same species, *T. cuspidata*. A possible explanation may be related to the somaclonal variation that is an inherent part of any plant cell culture system (Larkin and Scowcroft, 1981). Somaclonal variation has long been recognized to play a large role in the production of genetic variability within plant cell culture lines, with duplication or deletion of genetic elements representing possible causes. In particular, amino acid changes in coding regions may occur, leading to significant somaclonal variation. In the case of glufosinate resistance, it has been previously demonstrated that a single histidine to tyrosine substitution is sufficient to increase binding specificity of glutamine synthetase for glutamate, effectively increasing glufosinate resistance in soybean (Pornprom et al., 2009). Such an amino acid substitution may have occurred among the different *Taxus* cell lines as the result of somaclonal variation, resulting in the differential sensitivities to glufosinate observed.

Despite sensitivity of all examined *Taxus* cell lines to hygromycin B, it is often beneficial for a larger selection of antibiotics to be available as selectable markers when performing the directed metabolic engineering of any plant species. Oftentimes, once an effective *Agrobacterium*-mediated transformation protocol is established, the most limiting factor in the number of transgenes that can be introduced is the number of available selecting agents if no method exists for the *in planta* excision of selectable markers (e.g., Cre/lox site-specific recombination system). In regards to general kanamycin (R. Ketchum, personal communication) and glufosinate ammonium resistance observed in *Taxus* cell lines P991, P93AF, PO93X, and CO93D, chemical analogs may provide more success with incorporation of those resistance genes into binary transformation vectors. Geneticin, a chemical analog of kanamycin, has been shown to

increase plant selection efficiency when kanamycin was not effective (Radke et al., 1992).

If chemical analogs of kanamycin and glufosinate ammonium do not prove successful, many selective agents may still be utilized in the generation of transgenic *Taxus* cell lines. These selective agents range from various antibiotics (e.g., spectinomycin, streptomycin, etc.) to herbicides (e.g., glyphosate, Methionine sulfoximine, etc.) to metabolic analogs (e.g., 2-deoxyglucose) (Sundar and Sakthivel, 2008b).

5.4.2 Prospectives for the Generation of Transgenic *Taxus* Cell Lines

Traditional liquid-liquid co-cultivation systems (without or with *Agrobacterium* virulence gene priming) did not yield any positive *Taxus* transformants. In spite of a published report describing the amenability of *Taxus* cell lines P93AF and PO93X to traditional liquid-liquid *Agrobacterium* co-cultivation (Ketchum et al., 2007), this method was not reproducible by the paclitaxel research group at UMass Amherst. Despite sharing a common origin, the P93AF and PO93X cell lines were maintained independently by Ketchum et al. (2007), and likely developed characteristics specific to the conditions in their laboratory. This is not uncommon with aged plant cell lines that are maintained within the same general parameters; differences develop as the result of unspecified and sometimes minor changes, both over time and over distances. An example is the difference in ploidy level over time in *Taxus* culture; even within an individual laboratory, genome size of cultures varies greatly, and it is likely that this observed difference in genome size is exaggerated when *Taxus* cell lines are maintained by different research groups (Kim et al., 2004; Mardamshin et al., 2001).

Dry co-cultivation yielded positive transformants utilizing *Taxus* cell line PO93X and *Agrobacterium* strain EHA105 (containing 35S:intron-GUS) (Figure 5.7). The successful observation of transformation following dry co-cultivation has been reported in other recalcitrant plant species, such as wheat (Cheng and Fry, 2006). The explanation for the success of this method in situations where the more traditional liquid-liquid co-cultivation fails is largely unexplained, though it may relate to the reduced osmotic potential of the plant cell. It has previously been suggested that this reduced plant cell turgidity minimizes the damage to the cell wall caused by infection; this suggestion is speculative, however, and no extensive experiments have been conducted to uncover the mechanism underlying this association (Yin and Zhang, 2010). Additionally, several other factors may be related to the capacity of dry co-cultivation to facilitate T-DNA transfer from *Agrobacterium* strain EHA105 to *Taxus* cell line PO93X. Two of these factors are the absence of sucrose and NAA from the co-cultivation, two compounds that would normally be present in the Gamborg B5 media utilized in liquid-liquid co-cultivation. Sucrose has previously been demonstrated to be inhibitory to *Agrobacterium* virulence gene induction (Gelvin, 2006). While NAA has not been examined with regard to *Agrobacterium* virulence gene inhibition, IAA, an NAA analog, has been previously shown to inhibit virulence gene induction (Liu and Nester, 2006). The absence of these potential virulence gene inhibiting compounds, in conjunction with the dry co-cultivation, may have both facilitated the competency of *Taxus* cell line PO93X to accept T-DNA transfer from *Agrobacterium* strain EHA105.

The combination of *Taxus* cell line PO93X and *Agrobacterium* strain EHA105 during dry co-cultivation was also successful in yielding positive transformants in the

method described by Ketchum et al. (2007). Interestingly, *Taxus* cell line P991 was not amenable to dry co-cultivation with EHA105, despite originating from *T. cuspidata*, the same *Taxus* species that also yielded the less recalcitrant PO93X. A potential explanation for this differential host/pathogen interaction may be related to the differential taxane profiles for each line. *Taxus* cell line P991 is generally regarded as a reliable taxane producer, producing both paclitaxel and its intermediates. *Taxus* cell line PO93X, however, is generally produces very little taxanes. This pattern of taxane accumulation is likely a reflection of specific plant cell line immune response, as paclitaxel may be a plant defense compound. Macroarray data has verified the weak immune response to methyl jasmonate elicitation in PO93X (E. Walker, unpublished data), and suggests that this observed weak immune response may have affected the susceptibility of PO93X to *Agrobacterium* infection. This suggestion is further reinforced by the observation that a plant host immune response is attenuated during and following *Agrobacterium*-mediated transformation of a plant host, implying that a strong immune response is not advantageous in the horizontal transfer of transgenes by *Agrobacterium* (Gelvin, 2009; Yuan et al., 2007).

Numerous parameters may be optimized in this procedure, leading to increased transformation efficiency. The length of co-cultivation, the length of the recovery period, and the volume (though not necessarily optical density) of the *Agrobacterium* co-cultivated with the *Taxus* cells may yield an increase in *Agrobacterium* transformation efficiency. In addition, the screening of different *Taxus* cell lines may yield additional genotypes more amenable to *Agrobacterium*-mediated transformation, as indicated by

the significant role of an undescribed genetic factor controlling the capacity of *Taxus* cells to receive T-DNA from an *Agrobacterium* pathogen.

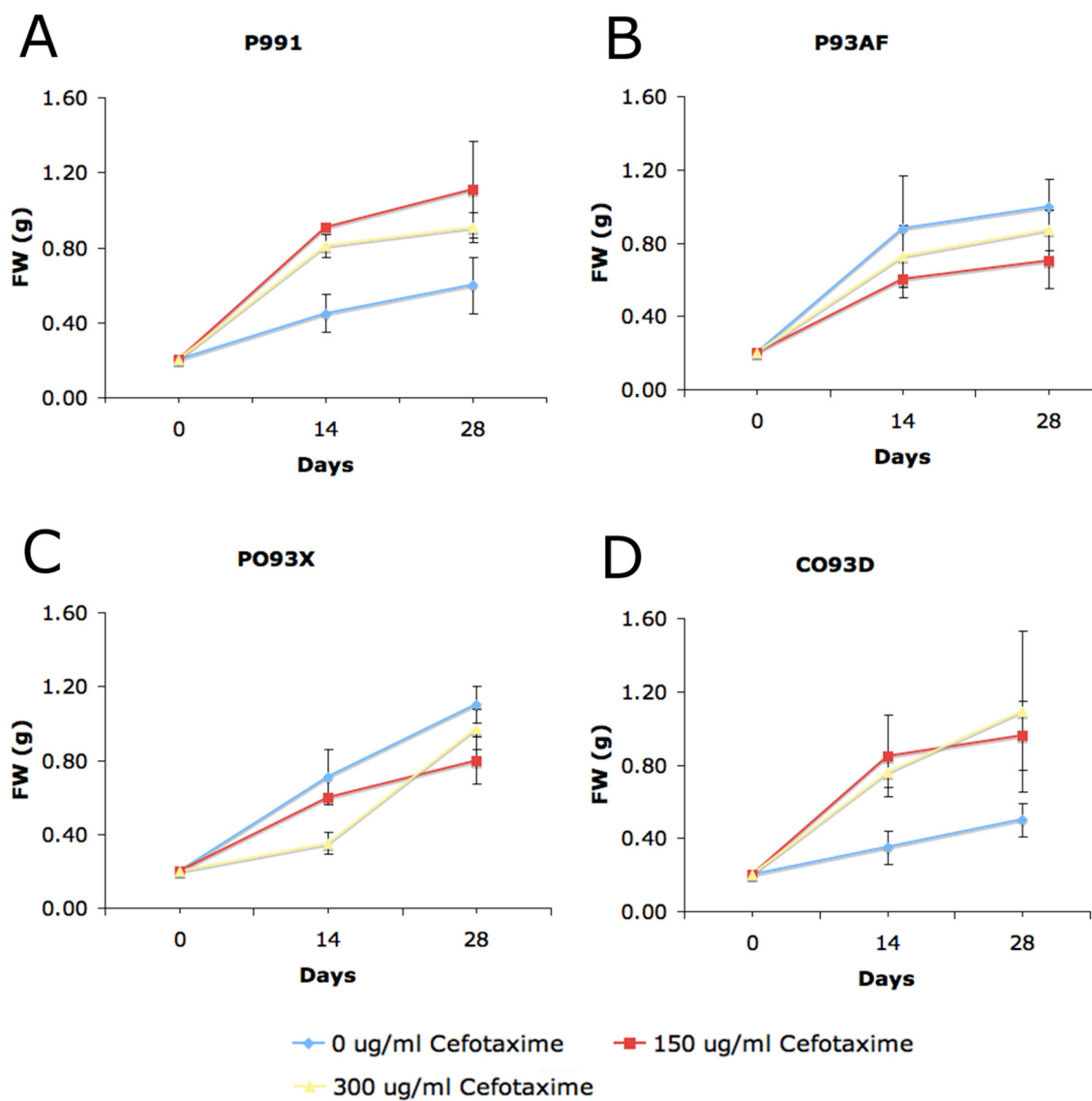


Figure 5.1: *Taxus* cell line viability on 0, 150, or 300 $\mu\text{g/ml}$ of cefotaxime following a 28-day incubation on solid Gamborg B5 media

(A) *Taxus* cell line P991, (B) *Taxus* cell line P93AF, (D) *Taxus* cell line PO93X, and (D) *Taxus* cell line CO93D.

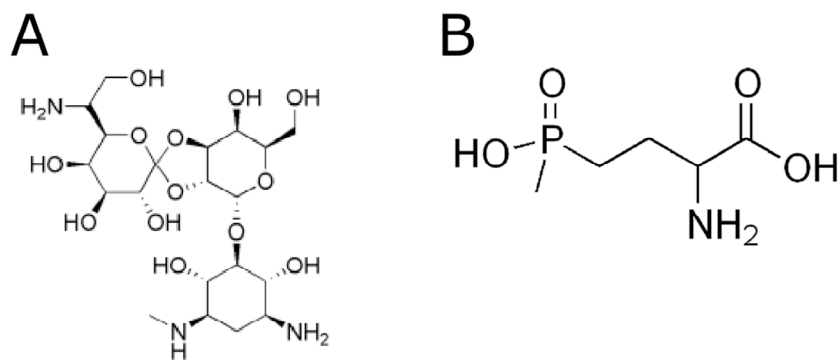


Figure 5.2: Molecular structure of hygromycin B and glufosinate

(A) represents hygromycin B, and (B) represents glufosinate.

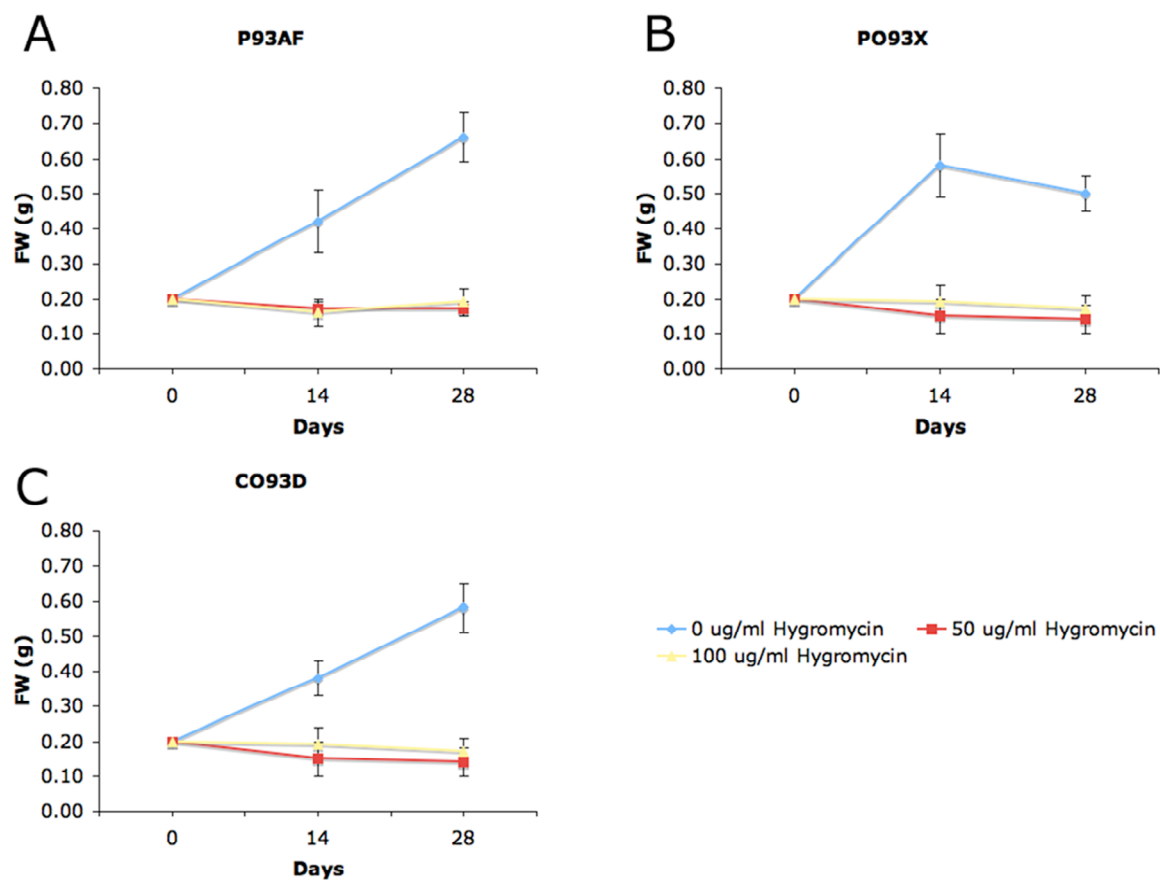


Figure 5.3: *Taxus* cell line viability on various concentrations of hygromycin B following a 28-day incubation on solid Gamborg B5 media

(A) *Taxus* cell line P93AF, (B) *Taxus* cell line PO93X, and (C) *Taxus* cell line CO93D incubated on 0, 50, and 100 $\mu\text{g/ml}$ hygromycin.

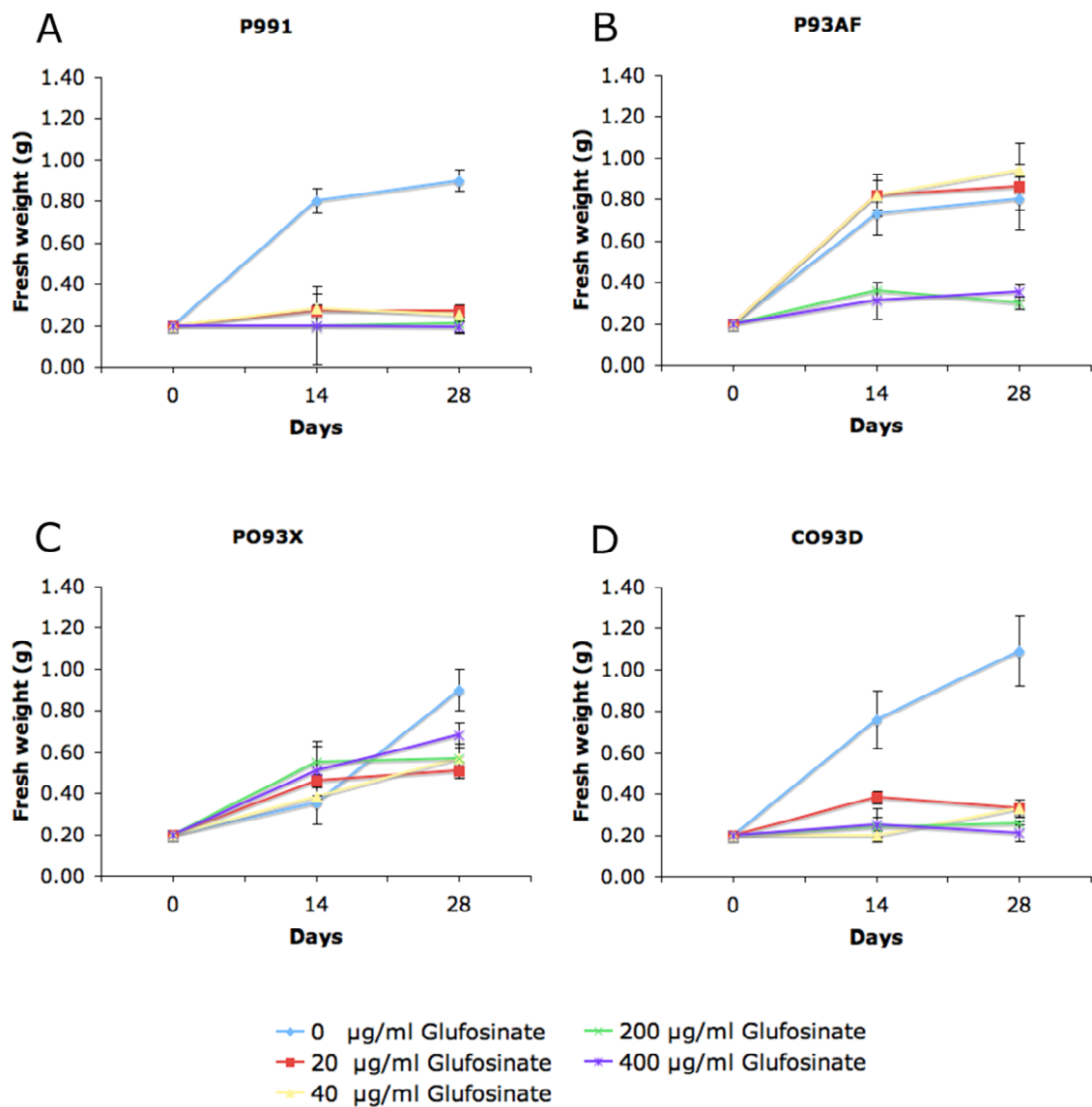


Figure 5.4: *Taxus* cell line viability on various concentrations of glufosinate following a 28-day incubation on solid Gamborg B5 media

Taxus cell line P991, (B) *Taxus* cell line P93AF, (D) *Taxus* cell line PO93X, and (D) *Taxus* cell line CO93D incubated on 0, 20, 40, 200, and 400 µg/ml glufosinate.

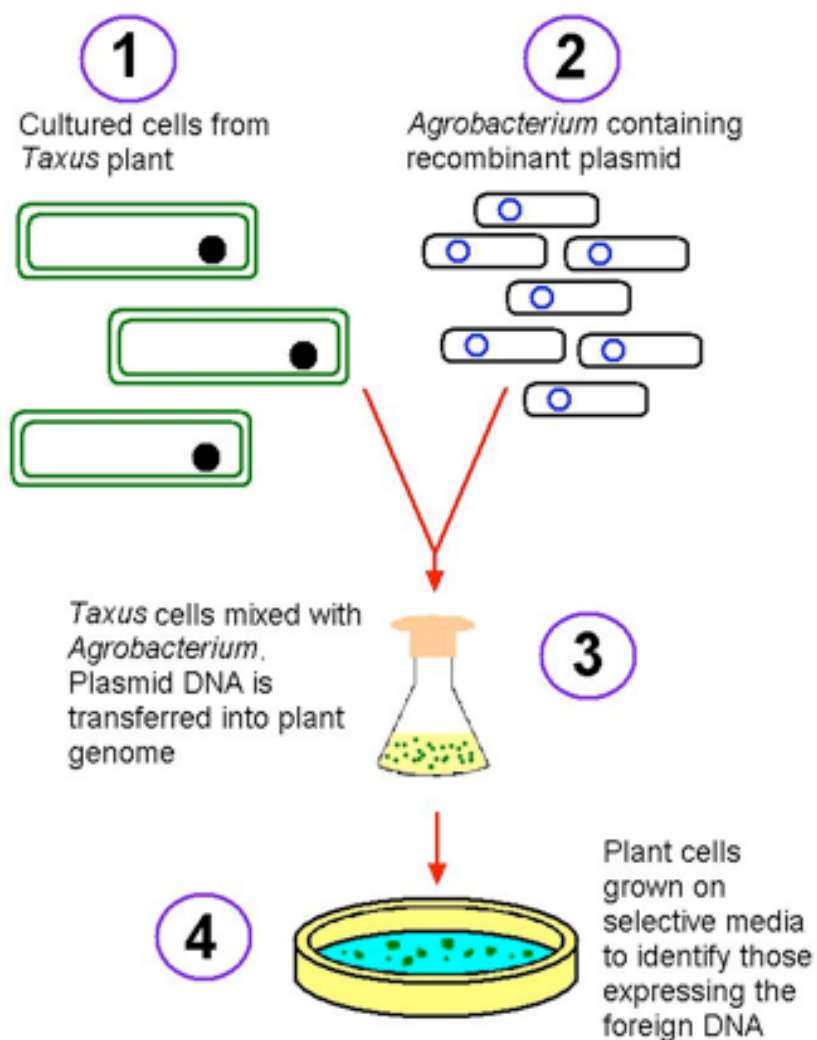


Figure 5.5: Standard liquid/liquid co-cultivation between *Taxus* and *Agrobacterium tumefaciens*

Parameters that can be manipulated include: (1) *Taxus* cell lines P991, P93AF, PO93X, and CO93D; (2a) *Agrobacterium* strains GV3101, LBA4404, EHA105, and C56C1; (2b) the reporter constructs 35S:GFP, 35S:GUS, 35S:intron-GUS; (2c) acetosyringone concentrations 0—100 μ M; (3) one or two day co-cultivation period; and (4) one to seven day recovery period in the dark at room temperature.

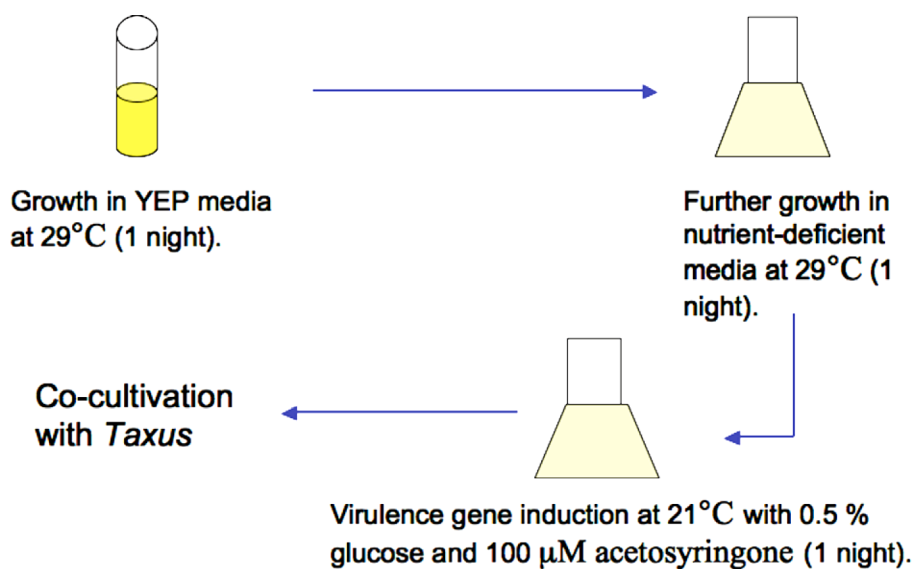


Figure 5.6: Priming of *Agrobacterium tumefaciens* prior to *Taxus* co-cultivation

The protocol (adapted from Gelvin, 2006) temporally separates *A. tumefaciens* vegetative growth (2 nights of growth, first in YEP media followed by growth in nutrient-deficient media) and virulence gene induction with glucose and acetosyringone (one night on glucose media).

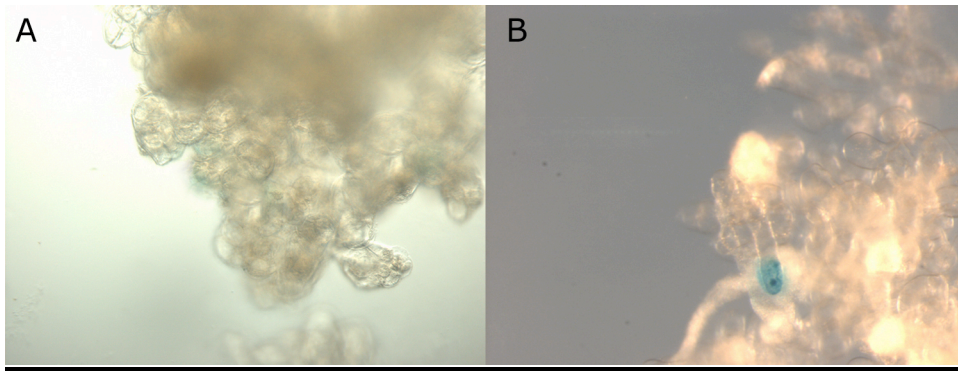


Figure 5.7: GUS activity in *Taxus* cell line PO93X following dry co-cultivation

Taxus cell line PO93X showing (A) no histochemical GUS activity and (B) histochemical GUS activity after a 72-hour solid co-cultivation period with *Agrobacterium tumefaciens* strain EHA105 (containing pCAMBIA1301) and a 48-hour recovery period.

Table 5.1: Media composition and protocol for Gelvin *Agrobacterium* virulence gene priming (Gelvin, 2006)

YEP Media (per liter)	
10 g	Peptone
10 g	Yeast Extract
5 g	NaCl
*it is not necessary before auto clave	

AB Sucrose Media (per liter)	
0.50%	Sucrose
50 ml	Sterile 20X AB buffer
50 ml	Sterile 20X AB Salts
900 ml	Nanopure

Induction Media (per liter)	
0.50%	Glucose
50 ml	AB Salts
2 mM	Phosphate buffer (pH 5.6)
50 mM	MES
100 μ M	Acetosyringone

20X AB Buffer (per liter)	
60 g	K ₂ HPO ₄
20 g	NaH ₂ PO ₄
*pH to 7.0 before autoclaving	

20X AB Salts (per liter)	
20 g	NH ₄ Cl
6 g	MgSO ₄ 7H ₂ O
3 g	KCl
0.2 g	CaCl ₂
0.05 g	FeSO ₄ 7H ₂ O

Table 5.2: Combination of factors related to the traditional liquid-liquid co-cultivation of *Taxus* spp. cell culture and *Agrobacterium tumefaciens*. Co-cultivations were undertaken utilizing *Taxus* cells 7-9 days post-subculture. Each attempt is measured as an independent co-cultivation consisting of three replicates.

Agro-bacterium strain	<i>Taxus</i> cell line	Construct	Aceto-syringone (μM)	Co-cultivation period (days)	Recovery period (days)	Attempts
GV3101	P991	35S:GUS	0	1	1	x2
GV3101	P991	35S:GUS	10	1	1	x2
GV3101	P991	35S:GUS	100	1	1	x2
GV3101	P991	35S:GUS	0	2	1	x2
GV3101	P991	35S:GUS	10	2	1	x2
GV3101	P991	35S:GUS	100	2	1	x2
GV3101	P991	35S:GUS	0	1	3	x2
GV3101	P991	35S:GUS	10	1	3	x2
GV3101	P991	35S:GUS	100	1	3	x2
GV3101	P991	35S:GUS	0	2	3	x2
GV3101	P991	35S:GUS	10	2	3	x2
GV3101	P991	35S:GUS	100	2	3	x2
GV3101	P991	35S:GUS	0	1	7	x2
GV3101	P991	35S:GUS	10	1	7	x2
GV3101	P991	35S:GUS	100	1	7	x2
GV3101	P991	35S:GUS	0	2	7	x2
GV3101	P991	35S:GUS	10	2	7	x2
GV3101	P991	35S:GUS	100	2	7	x2
GV3101	P93AF	35S:GUS	0	1	1	x2
GV3101	P93AF	35S:GUS	10	1	1	x2
GV3101	P93AF	35S:GUS	100	1	1	x2
GV3101	P93AF	35S:GUS	0	2	1	x2
GV3101	P93AF	35S:GUS	10	2	1	x2
GV3101	P93AF	35S:GUS	100	2	1	x2
GV3101	P93AF	35S:GUS	0	1	3	x2
GV3101	P93AF	35S:GUS	10	1	3	x2
GV3101	P93AF	35S:GUS	100	1	3	x2
GV3101	P93AF	35S:GUS	0	2	3	x2
GV3101	P93AF	35S:GUS	10	2	3	x2
GV3101	P93AF	35S:GUS	100	2	3	x2
GV3101	P93AF	35S:GUS	0	1	7	x2
GV3101	P93AF	35S:GUS	10	1	7	x2
GV3101	P93AF	35S:GUS	100	1	7	x2
GV3101	P93AF	35S:GUS	0	2	7	x2
GV3101	P93AF	35S:GUS	10	2	7	x2
GV3101	P93AF	35S:GUS	100	2	7	x2
GV3101	PO93X	35S:GUS	0	1	1	x2
GV3101	PO93X	35S:GUS	10	1	1	x2

Table 5.2 continued.

Agro-bacterium strain	<i>Taxus</i> cell line	Construct	Aceto-syringone (μM)	Co-cultivation period (days)	Recovery period (days)	Attempts
GV3101	PO93X	35S:GUS	100	1	1	x2
GV3101	PO93X	35S:GUS	0	2	1	x2
GV3101	PO93X	35S:GUS	10	2	1	x2
GV3101	PO93X	35S:GUS	100	2	1	x2
GV3101	PO93X	35S:GUS	0	1	3	x2
GV3101	PO93X	35S:GUS	10	1	3	x2
GV3101	PO93X	35S:GUS	100	1	3	x2
GV3101	PO93X	35S:GUS	0	2	3	x2
GV3101	PO93X	35S:GUS	10	2	3	x2
GV3101	PO93X	35S:GUS	100	2	3	x2
GV3101	PO93X	35S:GUS	0	1	7	x2
GV3101	PO93X	35S:GUS	10	1	7	x2
GV3101	PO93X	35S:GUS	100	1	7	x2
GV3101	PO93X	35S:GUS	0	2	7	x2
GV3101	PO93X	35S:GUS	10	2	7	x2
GV3101	PO93X	35S:GUS	100	2	7	x2
GV3101	CO93D	35S:GUS	0	1	1	x2
GV3101	CO93D	35S:GUS	10	1	1	x2
GV3101	CO93D	35S:GUS	100	1	1	x2
GV3101	CO93D	35S:GUS	0	2	1	x2
GV3101	CO93D	35S:GUS	10	2	1	x2
GV3101	CO93D	35S:GUS	100	2	1	x2
GV3101	CO93D	35S:GUS	0	1	3	x2
GV3101	CO93D	35S:GUS	10	1	3	x2
GV3101	CO93D	35S:GUS	100	1	3	x2
GV3101	CO93D	35S:GUS	0	2	3	x2
GV3101	CO93D	35S:GUS	10	2	3	x2
GV3101	CO93D	35S:GUS	100	2	3	x2
GV3101	CO93D	35S:GUS	0	1	7	x2
GV3101	CO93D	35S:GUS	10	1	7	x2
GV3101	CO93D	35S:GUS	100	1	7	x2
GV3101	CO93D	35S:GUS	0	2	7	x2
GV3101	CO93D	35S:GUS	10	2	7	x2
GV3101	CO93D	35S:GUS	100	2	7	x2
LBA4404	P991	35S:GUS	0	1	1	x1
LBA4404	P991	35S:GUS	10	1	1	x1
LBA4404	P991	35S:GUS	100	1	1	x1
LBA4404	P991	35S:GUS	0	2	1	x1
LBA4404	P991	35S:GUS	10	2	1	x1
LBA4404	P991	35S:GUS	100	2	1	x1
LBA4404	P991	35S:GUS	0	1	3	x1
LBA4404	P991	35S:GUS	10	1	3	x1

Table 5.2 continued.

Agro-bacterium strain	<i>Taxus</i> cell line	Construct	Aceto-syringone (μM)	Co-cultivation period (days)	Recovery period (days)	Attempts
LBA4404	P991	35S:GUS	100	1	3	x1
LBA4404	P991	35S:GUS	0	2	3	x1
LBA4404	P991	35S:GUS	10	2	3	x1
LBA4404	P991	35S:GUS	100	2	3	x1
LBA4404	P991	35S:GUS	0	1	7	x1
LBA4404	P991	35S:GUS	10	1	7	x1
LBA4404	P991	35S:GUS	100	1	7	x1
LBA4404	P991	35S:GUS	0	2	7	x1
LBA4404	P991	35S:GUS	10	2	7	x1
LBA4404	P991	35S:GUS	100	2	7	x1
LBA4404	P93AF	35S:GUS	0	1	1	x1
LBA4404	P93AF	35S:GUS	10	1	1	x1
LBA4404	P93AF	35S:GUS	100	1	1	x1
LBA4404	P93AF	35S:GUS	0	2	1	x1
LBA4404	P93AF	35S:GUS	10	2	1	x1
LBA4404	P93AF	35S:GUS	100	2	1	x1
LBA4404	P93AF	35S:GUS	0	1	3	x1
LBA4404	P93AF	35S:GUS	10	1	3	x1
LBA4404	P93AF	35S:GUS	100	1	3	x1
LBA4404	P93AF	35S:GUS	0	2	3	x1
LBA4404	P93AF	35S:GUS	10	2	3	x1
LBA4404	P93AF	35S:GUS	100	2	3	x1
LBA4404	P93AF	35S:GUS	0	1	7	x1
LBA4404	P93AF	35S:GUS	10	1	7	x1
LBA4404	P93AF	35S:GUS	100	1	7	x1
LBA4404	P93AF	35S:GUS	0	2	7	x1
LBA4404	P93AF	35S:GUS	10	2	7	x1
LBA4404	P93AF	35S:GUS	100	2	7	x1
LBA4404	PO93X	35S:GUS	0	1	1	x1
LBA4404	PO93X	35S:GUS	10	1	1	x1
LBA4404	PO93X	35S:GUS	100	1	1	x1
LBA4404	PO93X	35S:GUS	0	2	1	x1
LBA4404	PO93X	35S:GUS	10	2	1	x1
LBA4404	PO93X	35S:GUS	100	2	1	x1
LBA4404	PO93X	35S:GUS	0	1	3	x1
LBA4404	PO93X	35S:GUS	10	1	3	x1
LBA4404	PO93X	35S:GUS	100	1	3	x1
LBA4404	PO93X	35S:GUS	0	2	3	x1
LBA4404	PO93X	35S:GUS	10	2	3	x1
LBA4404	PO93X	35S:GUS	100	2	3	x1
LBA4404	PO93X	35S:GUS	0	1	7	x1
LBA4404	PO93X	35S:GUS	10	1	7	x1

Table 5.2 continued.

Agro-bacterium strain	<i>Taxus</i> cell line	Construct	Aceto-syringone (μM)	Co-cultivation period (days)	Recovery period (days)	Attempts
LBA4404	PO93X	35S:GUS	100	1	7	x1
LBA4404	PO93X	35S:GUS	0	2	7	x1
LBA4404	PO93X	35S:GUS	10	2	7	x1
LBA4404	PO93X	35S:GUS	100	2	7	x1
LBA4404	CO93D	35S:GUS	0	1	1	x1
LBA4404	CO93D	35S:GUS	10	1	1	x1
LBA4404	CO93D	35S:GUS	100	1	1	x1
LBA4404	CO93D	35S:GUS	0	2	1	x1
LBA4404	CO93D	35S:GUS	10	2	1	x1
LBA4404	CO93D	35S:GUS	100	2	1	x1
LBA4404	CO93D	35S:GUS	0	1	3	x1
LBA4404	CO93D	35S:GUS	10	1	3	x1
LBA4404	CO93D	35S:GUS	100	1	3	x1
LBA4404	CO93D	35S:GUS	0	2	3	x1
LBA4404	CO93D	35S:GUS	10	2	3	x1
LBA4404	CO93D	35S:GUS	100	2	3	x1
LBA4404	CO93D	35S:GUS	0	1	7	x1
LBA4404	CO93D	35S:GUS	10	1	7	x1
LBA4404	CO93D	35S:GUS	100	1	7	x1
LBA4404	CO93D	35S:GUS	0	2	7	x1
LBA4404	CO93D	35S:GUS	10	2	7	x1
LBA4404	CO93D	35S:GUS	100	2	7	x1
C56C1	P991	35S:intron-GUS	100	1	2	x3
C56C1	P93AF	35S:intron-GUS	100	1	2	x3
C56C1	PO93X	35S:intron-GUS	100	1	2	x3
C56C1	CO93D	35S:intron-GUS	100	1	2	x3

Table 5.3: Combination of factors related to reproduction of the *Agrobacterium*-mediated transformation protocol stated in Ketchum et al (2007). Co-cultivations were undertaken utilizing *Taxus* cells 7-9 days post-subculture. Each attempt is measured as an independent co-cultivation consisting of three replicates.

Agro-bacterium strain	<i>Taxus</i> cell line	Construct	Sonication (seconds)	Attempts
EHA105	P991	35S:intron-GUS	0	x4
EHA105	P991	35S:intron-GUS	30	x1
EHA105	P991	35S:intron-GUS	60	x1
EHA105	P93AF	35S:intron-GUS	0	x4
EHA105	P93AF	35S:intron-GUS	30	x1
EHA105	P93AF	35S:intron-GUS	60	x1
EHA105	PO93X	35S:intron-GUS	0	x4
EHA105	PO93X	35S:intron-GUS	30	x1
EHA105	PO93X	35S:intron-GUS	60	x1
EHA105	CO93D	35S:intron-GUS	0	x4
EHA105	CO93D	35S:intron-GUS	30	x1
EHA105	CO93D	35S:intron-GUS	60	x1

Table 5.4: Combination of factors related to the traditional liquid-liquid co-cultivation of *Taxus* spp. cell culture and *Agrobacterium tumefaciens* following the priming of *Agrobacterium* virulence genes as described in Gelvin (2005). Co-cultivations were undertaken utilizing *Taxus* cells 7-9 days post-subculture. Each attempt is measured as an independent co-cultivation consisting of three replicates.

Agro-bacterium strain	<i>Taxus</i> cell line	Construct	Aceto-syringone (µM)	Co-cultivation period (days)	Recovery period (days)	Attempts
GV3101	P991	35S:intron-GUS	100	1	2	x3
GV3101	P991	35S:mCherry	100	1	2	x3
GV3101	P93AF	35S:intron-GUS	100	1	2	x3
GV3101	P93AF	35S:mCherry	100	1	2	x3
GV3101	PO93X	35S:intron-GUS	100	1	2	x3
GV3101	PO93X	35S:mCherry	100	1	2	x3
GV3101	CO93D	35S:intron-GUS	100	1	2	x3
GV3101	CO93D	35S:mCherry	100	1	2	x3
LBA4404	P991	35S:intron-GUS	100	1	2	x3
LBA4404	P991	35S:mCherry	100	1	2	x3
LBA4404	P93AF	35S:intron-GUS	100	1	2	x3
LBA4404	P93AF	35S:mCherry	100	1	2	x3
LBA4404	PO93X	35S:intron-GUS	100	1	2	x3
LBA4404	PO93X	35S:mCherry	100	1	2	x3
LBA4404	CO93D	35S:intron-GUS	100	1	2	x3
LBA4404	CO93D	35S:mCherry	100	1	2	x3
EHA105	P991	35S:intron-GUS	100	1	2	x3
EHA105	P991	35S:mCherry	100	1	2	x3
EHA105	P93AF	35S:intron-GUS	100	1	2	x3

Table 5.4 continued.

Agro-bacterium strain	<i>Taxus</i> cell line	Construct	Aceto-syringone (μM)	Co-cultivation period (days)	Recovery period (days)	Attempts
EHA105	P93AF	35S: mCherry	100	1	2	x3
EHA105	PO93X	35S:intron-GUS	100	1	2	x3
EHA105	PO93X	35S: mCherry	100	1	2	x3
EHA105	CO93D	35S:intron-GUS	100	1	2	x3
EHA105	CO93D	35S: mCherry	100	1	2	x3
C56C1	P991	35S:intron-GUS	100	1	2	x3
C56C1	P991	35S: mCherry	100	1	2	x3
C56C1	P93AF	35S:intron-GUS	100	1	2	x3
C56C1	P93AF	35S: mCherry	100	1	2	x3
C56C1	PO93X	35S:intron-GUS	100	1	2	x3
C56C1	PO93X	35S: mCherry	100	1	2	x3
C56C1	CO93D	35S:intron-GUS	100	1	2	x3
C56C1	CO93D	35S: mCherry	100	1	2	x3

Table 5.5: Combination of factors related to the dry co-cultivation of *Taxus* spp. cell culture and *Agrobacterium tumefaciens* following the priming of *Agrobacterium* virulence genes as described in Gelvin (2005). Each attempt is measured as an independent co-cultivation consisting of three replicates.

Agro-bacterium strain	<i>Taxus</i> cell line	Construct	Aceto-syringone (µM)	Co-cultivation period (days)	Recovery period (days)	Attempts
GV3101	P991	35S:intron-GUS	100	2	2	x1
GV3101	P93AF	35S:intron-GUS	100	2	2	x3
GV3101	PO93X	35S:intron-GUS	100	2	2	x3
GV3101	CO93D	35S:intron-GUS	100	2	2	x1
LBA4404	P991	35S:intron-GUS	100	2	2	x1
LBA4404	P93AF	35S:intron-GUS	100	2	2	x3
LBA4404	PO93X	35S:intron-GUS	100	2	2	x3
LBA4404	CO93D	35S:intron-GUS	100	2	2	x1
EHA105	P991	35S:intron-GUS	100	2	2	x1
EHA105	P93AF	35S:intron-GUS	100	2	2	x3
EHA105	PO93X	35S:intron-GUS	100	2	2	x3
EHA105	CO93D	35S:intron-GUS	100	2	2	x1
C56C1	P991	35S:intron-GUS	100	2	2	x1
C56C1	P93AF	35S:intron-GUS	100	2	2	x3
C56C1	PO93X	35S:intron-GUS	100	2	2	x3
C56C1	CO93D	35S:intron-GUS	100	2	2	x1

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Introduction

Engineering of superior paclitaxel-accumulating *Taxus* cell lines requires a synthesis of knowledge and technologies spanning molecular biology and engineering. Identification of novel taxane structural genes and metabolic bottlenecks necessitates the use of tools and strategies to characterize these genes, as well as an *Agrobacterium*-mediated transformation protocol to stably introduce these genes into *Taxus* for long-term evaluation of gene overexpression/silencing on taxane accumulation profiles. Novel methods were developed for the characterization of taxane-related genes from *Taxus*, and transgene introduction was achieved through *Agrobacterium tumefaciens*, demonstrating a viable method for optimization of stable transgene integration.

6.2 Transient Introduction of Transgenes Through Particle Bombardment

In Chapter 3, a method for the particle bombardment-mediated transient transformation in the P991 and CO93D *Taxus* cell lines was developed. Utilizing the firefly luciferase gene as a reporter, several parameters were optimized for transient expression in P991 and CO93D. The optimized parameters shared between the two transformable *Taxus* cell lines include a 6 cm target distance, an 1100 psi rupture disc pressure, utilization of gold microcarriers, a 1.6 μm microcarrier diameter, and multiple bombardments/target. Optimization of plasmid and gold microcarrier concentration was cell line dependent. *Taxus* cell line P991 utilized 1 μg of plasmid DNA and 60 mg/ml of

gold microcarriers for maximal transient luciferase expression, while bombardments with *Taxus* cell line CO93D required 2 µg of plasmid DNA and 120 mg/ml of gold microcarriers for maximal transient luciferase expression.

An advantage of this transient transformation system is the potential for relatively quick and efficient characterization of novel *Taxus* genes, allowing for transformation with multiple gene constructs. For example, analysis of putative paclitaxel biosynthetic pathway regulators can be achieved through co-bombardments with the transcriptional regulator and paclitaxel biosynthetic gene promoter/GUS fusion constructs (*Nims et al.*, unpublished data). In addition, direct localization of paclitaxel biosynthetic genes can be achieved after particle bombardment with paclitaxel biosynthetic gene/DsRed fusion constructs. These transient transformation studies can be completed within days as opposed to waiting for enough biomass for stable transformation *that* can take months.

While particle bombardment was intended as a transient transformation method to characterize putative paclitaxel biosynthesis regulatory genes, this does not preclude the possibility of utilizing this method to generate stably transformed *Taxus* cell lines. Particle bombardment has generated stable transformants in many recalcitrant monocots and gymnosperms, though complex and multiple insertion events are typical (Lowe et al., 2009). In the case of experiments described in this thesis, the bombarded *Taxus* cells often underwent necrosis 10-14 days following bombardment, a period of time coinciding with recovery in systems generating stable transformants. Utilizing different recovery media may be useful in promoting growth of *Taxus* cells following particle bombardment. In addition, further examination of different selectable markers may be advantageous in the selection of positive transformants. *Taxus* has shown a wide

tolerance for selectable markers, being extremely resistant to kanamycin and glufosinate. While susceptibility to gentamycin and hygromycin has been validated for *Taxus* cells in culture, growth of *Taxus* cells following selection may benefit from additional examination of existing antibiotic analogues, such as the kanamycin analogue geneticin, due to slight differences in structure that may ultimately affect analogue binding and selection efficiency (Sundar and Sakthivel, 2008a). In addition, other selection mechanisms may also be employed, such as utilizing the enhanced expression of an *A. thaliana* tryptophan synthase beta gene and the selective agent 5-methyl-tryptophan. Utilizing a variety of selection systems may facilitate the selection of positive transformants, as non-transformed *Taxus* cells may not immediately undergo necrosis, reducing the tendency isolate transformed cells as “cell islands” and allowing greater access of these positive transformants to chemical crosstalk necessary for mitotic division and generation of calli containing positive transformants.

6.2 Agroinfiltration of *Nicotiana benthamiana* with Taxadiene Synthase and Taxadiene-5 α -hydroxylase

An agroinfiltration system in *Nicotiana benthamiana* was developed for the examination of taxane biosynthetic genes. Central to this methodology were three factors: a) the high transformation efficiency of the *N. benthamiana* agroinfiltration system, b) the capacity this agroinfiltration system to undergo co-transformation of individual cells by different populations of *Agrobacterium*, each containing a different gene in a single biosynthetic pathway, and c) the substrate plasticity of the taxane CYP450s, such as taxadiene-5 α -hydroxylase. The third factor is of greatest importance to the remaining uncloned and uncharacterized putative taxane CYP405s isolated in our

laboratory. Lack of substrates has been a limiting factor in the characterization of taxane biosynthetic genes, forcing researchers to utilize sparsely-available dead-end or synthetic taxanes (e.g., [+-] taxusin) in non-plant characterization systems, such as yeast or Sf9 insect cells. However, utilizing the capacity of this *N. benthamiana* system to undergo co-transformation of individual mesophyll cells, reconstruction of base taxane substrates can be achieved *in planta*, perhaps allowing their use in the characterization of novel taxane CYP450s.

Agroinfiltration of taxadiene synthase (TASY) into the leaves of *N. benthamiana* yielded of taxa-4(5), 11(12)-diene from extracted plant tissue. The recovery of the first dedicated product of the taxane biosynthetic pathway demonstrates that native *N. benthamiana* GGPP pools are sufficient to drive metabolic flux into the introduced pathway. In addition, detrimental phenotypic effects are not observed, as was previously observed in *Arabidopsis thaliana* overexpressing TASY. However, when TASY is co-agroinfiltrated with taxadien-5 α -hydroxylase (T5 α H), neither taxa-4(5), 11(12)-diene or the expected taxa-4(20),11(12)-dien-5 α -ol are observed. Manipulating the amount of TASY relative to T5 α H (9:1 ratio) permitted the detection of taxa-4(5), 11(12)-diene, though no taxa-4(20),11(12)-dien-5 α -ol was detected. In addition, quantification of TASY:T5 α H co-agroinfiltration (9:1) to TASY:mCherry co-agroinfiltrations (9:1) demonstrated that the introduction of T5 α H into the agroinfiltration decreased taxa-4(5), 11(12)-diene concentrations. These two points demonstrate that T5 α H is acting upon the product of TASY, taxa-4(5), 11(12)-diene, and that it is either catalyzing a reaction that does not yield the expected taxa-4(20),11(12)-dien-5 α -ol, or that taxa-4(20),11(12)-dien-5 α -ol is being catabolized to another product natively by *N. benthamiana*. Additionally,

the extraction/purification method may not be sufficient to detect the product of T5 α H. To detect these products, a different extraction/purification method is required. The simplest solution would be to first utilize extraction solvents other than hexane, such as pentane or a pentane:ethyl acetate solution. Additionally, nuclear magnetic resonance (NMR) allows structural identification of novel compounds, in contrast to the mass spectroscopy (MS) used here in tandem with the gas chromatography (GC) separations unit. However, NMR requires pure compounds for analysis and not the total plant extract produced as a result of hexane extraction and solid phase extraction (SPE) purification. While this work was developed utilizing a GC-MS, these early taxane compounds can be analyzed through liquid chromatography mass spectroscopy (LC-MS), and differential peak profiles can be determined from TASY and TASY + T5 α H agroinfiltration extracts. Following identification of unique peaks, a fractionator attached downstream of the LC column can be used to collect samples based on retention time, with these samples being used as injection material for NMR analysis.

6.3 *Agrobacterium*-mediated Transformation of *Taxus*

Stable integration of plasmid DNA is the only method of examining the long term effects of gene overexpression/silencing in *Taxus* cell culture, in addition to being a necessary prerequisite towards the metabolic engineering of superior paclitaxel-accumulating cell lines. Compared to other methods of stable DNA integration, such as particle bombardment, *Agrobacterium*-mediated transformation results in a higher proportion of single transformation events, alleviating issues from multiple T-DNA insertions, such as complex insertion patterns and gene silencing.

Selection agent sensitivity was determined through a screen with several commonly used selection agents, including cefotaxime, hygromycin B, and glufosinate. Though different *Taxus* cells exhibited varying degrees of sensitivity, overall trends included the absence of resistance of all four evaluated *Taxus* cell lines cefotaxime and hygromycin B at commonly-used concentrations. Analogues of antibiotic or herbicide selection compounds may facilitate the selection of positive transformants following transformation, as similar overall but different chemical moieties may affect selection efficiency.

Traditional liquid/liquid co-cultivation methods did not yield any positive transformants in this study, despite the manipulation of numerous parameters and an alternative route of *Agrobacterium* virulence gene priming before co-cultivation. Additionally, a published method describing the *Agrobacterium*-mediated transformation of *Taxus* cells in culture was not reproducible. False positives of transformation were seen in the *Taxus* system, as demonstrated by stress autofluorescence resembling GFP or native plant GUS activity in chapter 3. However, methods were devised to alleviate these concerns, such as use of alternate reporter genes (e.g., pCAMBIA1301 containing 35S:intron-GUS) or increasing the pH of the GUS histochemical buffer to prevent plant GUS activity. Successful *Agrobacterium*-mediated transformation system was demonstrated in this study with *Taxus* cell line PO93X and *Agrobacterium tumefaciens* strain EHA105 utilizing a dry co-cultivation system. Histochemical β -glucuronidase activity was observed following a two-day dry co-cultivation with *A. tumefaciens* primed with a specific protocol designed to separate vegetative growth and virulence gene induction. During selection with hygromycin B, no growth of positive transformants was

observed, likely due to the low efficiency of transformation ($\leq 1\%$) of the method. Optimization of several addition parameters, such as volume of *Agrobacterium* applied during the co-cultivation, length of dry co-cultivation, and additional screening of additional *Taxus* cell lines for use during dry co-cultivation may increase the success of generating a stably transformed *Taxus* cell line following co-cultivation and selection. Probably most important of these recommended parameters for further study is the screening of additional *Taxus* cell lines for dry co-cultivation. Genotype-genotype interaction is of paramount importance to the successful uptake and integration of T-DNA from *Agrobacterium* to the plant host. Genetic response has been shown to be variable between plant cell lines utilized for *Agrobacterium*-mediated transformation, and was confirmed in this study and the published work describing the successful generation of transgenic *Taxus* cell lines (Ketchum et al., 2007). This result of this genotype-genotype interaction is documented repeatedly in the literature. Additional screening of *Taxus* cell lines for use in *Agrobacterium*-mediated transformation is likely the best tool for increasing transformation efficiencies, as well as identifying those elite *Taxus* cell lines that have a capacity for rapid regrowth following a successful transformation event.

6.4 Prospects of Metabolic Engineering in *Taxus* Cell Culture

Progress is being made towards the metabolic engineering of *Taxus* cells for increased paclitaxel accumulation. Once this successful demonstration of *Agrobacterium*-mediated transformation has been developed into a reliable protocol, overexpression/silencing of known paclitaxel biosynthetic structural genes should be among the first experiments undertaken. Two putative metabolic bottlenecks, DBAT and DBTNBT have been identified and overexpression of these two genes may lead to

increased accumulation of paclitaxel in cell culture (Nims et al., 2007). In addition, overexpression of a characterized MYC transcriptional regulator involved in the paclitaxel biosynthetic pathway may also lead to increased paclitaxel accumulation in cell culture (Nims et al., unpublished data).

Silencing of dead-end routes to paclitaxel may facilitate the shuffling of metabolic flux towards pathways and metabolites that are directly involved in the generation of paclitaxel. T14 β H is a taxane CYP450 that has been identified as the first dead-end taxane biosynthetic gene, as hydroxylation of C14 is not directly involved in the generation of the paclitaxel molecule. Other dead end structural genes are hypothesized to exist, as the detection of over 300 taxane metabolites indicates that several paclitaxel-related biosynthetic genes have yet to be cloned (Jennewein et al., 2003; Kaspera and Croteau, 2006).

It is prudent to mention that overexpression/silencing of regulatory genes through molecular biology is just one aspect in the metabolic engineering of superior paclitaxel-accumulating *Taxus* lines. Many other facets will need to be addressed in order to promote commercialization of elite *Taxus* cell lines. Fermentation aspects related to large-scale maintenance of *Taxus* cells in culture will have to be addressed, in addition to the variability over time of *Taxus* cell culture morphology and aggregation. Subpopulations representing differential regulation of cell cycle participation and differential paclitaxel accumulation have already been identified in small-scale cultures, and are anticipated to increase in complexity with larger cell culture volumes. Increasing the understanding of the physical and cellular mechanisms responsible for creation of

these subpopulations will be instrumental in the successful development and commercialization of elite paclitaxel-accumulating *Taxus* cell lines.

The metabolic engineering of *Taxus* cell culture is still only in its infancy. A lack of decrease in cancer detection rates compounded with an expanded application of paclitaxel against additional cancers and diseases will strain current supplies of this anti-mitotic agent. Increasing the absolute quantity of paclitaxel available for pharmaceutical purposes not only will match and sustain demand, but will also decrease the price of this drug.

BIBLIOGRAPHY

- Ajikumar, P., W-H. Xiao, K. Tyo, Y. Wang, F. Simeon, E. Leonard, O. Mucha, T. Phon, B. Pfeifer, and G. Stephanopolous. 2010. Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science* 330:70-74.
- Alves, S.C., B. Worland, V. Thole, J.W. Snape, M.W. Bevan, and P. Vain. 2009. A protocol for *Agrobacterium*-mediated transformation of *Brachypodium distachyon* community standard line Bd21. *Nat Protoc* 4:638-49.
- Baebler, S., M. Camloh, M. Kovac, M. Ravnkar, and J. Zel. 2002. Jasmonic acid stimulates taxane production in cell suspension culture of yew (*Taxus x media*). *Planta Med* 68:475-6.
- Balunas, M.J., and A.D. Kinghorn. 2005. Drug discovery from medicinal plants. *Life Sci* 78:431-441.
- Bennetzen, J.L., S. Hake, and A. Gierl. 2009. The Cytochrome P450 superfamily of monooxygenases, p. 731-739 *Handbook of Maize*. Springer New York.
- Besumbes, O., S. Sauret-Gueto, M.A. Phillips, S. Imperial, M. Rodriguez-Concepcion, and A. Boronat. 2004. Metabolic engineering of isoprenoid biosynthesis in *Arabidopsis* for the production of taxadiene, the first committed precursor of Taxol. *Biotechnol Bioeng* 88:168-175.
- Bhatnagar, S., A. Kapur, and P. Khurana. 2002. Evaluation of parameters for high efficiency gene transfer via particle bombardment in Indian mulberry. *Indian J Exp Biol* 40:1387-92.
- Bhau, B.S., and A.K. Wakhlu. 2001. Effect of Some Antibiotics on the In Vitro Morphogenetic Response from Callus Cultures of *Coryphantha Elephantidens*. *Biologia Plantarum* 44:19-24.
- Chapple, C. 1998. Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. *Annual Review of Plant Physiology* 49:311-343.
- Chau, M., and R. Croteau. 2004. Molecular cloning and characterization of a cytochrome P450 taxoid 2 alpha-hydroxylase involved in taxol biosynthesis. *Arch Biochem Biophys* 427:48-57.
- Chau, M., S. Jennewein, K. Walker, and R. Croteau. 2004. Taxol biosynthesis: Molecular cloning and characterization of a cytochrome p450 taxoid 7 beta-hydroxylase. *Chem Biol* 11:663-672.

- Cheng, M., and J. Fry. 2006. An improved *Agrobacterium*-mediated plant transformation protocol 2006. EP1137790B1.
- Choi, H.K., J.H. Yun, S.I. Kim, J.S. Son, H.R. Kim, J.H. Kim, H.J. Choi, and S.S. Hong. 2002. Enhanced production of paclitaxel by semi-continuous batch process (SCBP) in suspension culture of *Taxus chinensis* (vol 29, pg 583, 2001). *Enzyme Microb Technol* 31:368-368.
- Ciddi, V., V. Srinivasan, and M.L. Shuler. 1995. Elicitation of *Taxus* spp. cell cultures for production of taxol. *Biotechnol Lett* 17:1343-1346.
- Constantin, G.D., M. Gronlund, I.E. Johansen, J. Stougaard, and O.S. Lund. 2008. Virus-induced gene silencing (VIGS) as a reverse genetic tool to study development of symbiotic root nodules. *Mol Plant Microbe Interact* 21:720-7.
- Croteau, R.K., R.E.B.; Long, R.M.; Kaspera, R.; and Wildung, M.R. 2006. Taxol biosynthesis and molecular genetics. *Phytochem Rev* 5:75-97.
- Cseke, L.J., S.B. Cseke, and G.K. Podila. 2007. High efficiency poplar transformation. *Plant Cell Rep* 26:1529-38.
- D'Aoust, M.A., P.O. Lavoie, J. Belles-Isles, N. Bechtold, M. Martel, and L.P. Vezina. 2009. Transient expression of antibodies in plants using syringe agroinfiltration. *Methods Mol Biol* 483:41-50.
- d'Utra Vaz, F.B., A.V.P.d. Santos, G. Manders, E.C. Cocking, M.R. Davey, and J.B. Power. 1993. Plant regeneration from leaf mesophyll protoplasts of the tropical woody plant, passion fruit: the importance of the antibiotic cefotaxime in the culture medium. *Plant Cell Rep* 12:220-225.
- Danilova, S.A., and Y.I. Dolgikh. 2004. The stimulatory effect of the antibiotic cefotaxime on plant regeneration in maize tissue culture. *Russian Journal of Plant Physiology* 51:559-562.
- Datta, M.M., A. Majumder, and S. Jha. 2006. Organogenesis and plant regeneration in *Taxus wallichiana* (Zucc.). *Plant Cell Rep* 25:11-8.
- Di Fiore, S., Q. Li, M.J. Leech, F. Schuster, N. Emans, R. Fischer, and S. Schillberg. 2002. Targeting tryptophan decarboxylase to selected subcellular compartments of tobacco plants affects enzyme stability and in vivo function and leads to a lesion-mimic phenotype. *Plant Physiol* 129:1160-9.
- Doraiswamy, P.M., and G. Xiong. 2006. Pharmacological strategies for the prevention of Alzheimer's disease. *Expert Opinion on Pharmacotherapy* 7:1-10.

- Dornenburg, H., and D. Knorr. 1995. Strategies for the improvement of secondary metabolite production in plant-cell cultures. *Enzyme Microb Technol* 17:674-684.
- Eisenreich, W., B. Menhard, P.J. Hylands, M.H. Zenk, and A. Bacher. 1996. Studies on the biosynthesis of taxol: The taxane carbon skeleton is not of mevalonoid origin. *Proc Natl Acad Sci U S A* 93:6431-6436.
- Exposito, O., M. Bonfill, E. Moyano, M. Onrubia, M.H. Mirjalili, R.M. Cusido, and J. Palazon. 2009a. Biotechnological production of taxol and related taxoids: current state and prospects. *Anticancer Agents Med Chem* 9:109-21.
- Exposito, O., K. Syklovska-Baranek, E. Moyano, M. Onrubia, M. Bonfill, J. Palazon, and R.M. Cusido. 2009b. Metabolic responses of *Taxus media* transformed cell cultures to the addition of methyl jasmonate. *Biotechnol Prog* 26:1145-1153.
- Fett-Neto, A.G. Pennington, and J.J. DiCosmo. 1995. Effect of white light on taxol and baccatin III accumulation in cell cultures of *Taxus cuspidata* Sieb and Zucc. *J Plant Physiol* 146:584-590.
- Fischer, R., C. Vaquero-Martin, M. Sack, J. Drossard, N. Emans, and U. Commandeur. 1999. Towards molecular farming in the future: transient protein expression in plants. *Biotechnol Appl Biochem* 30 (Pt 2):113-6.
- Frame, B.R., T. Paque, and K. Wang. 2006. Maize (*Zea mays* L.), p. 185-200, Vol. 343.
- Frense, D. 2007. Taxanes: perspectives for biotechnological production. *Appl Microbiol Biotechnol* 73:1233-40.
- Furmanowa, M., H. Oledzka, K. Syklovska-Baranek, J. Jozefowicz, and S. Gieracka. 2000. Increased taxane accumulation in callus cultures of *Taxus cuspidata* and *Taxus x media* by some elicitors and precursors. *Biotechnol Lett* 22:1449-1452.
- Gallagher, S.R. 1992. *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression*. Academic Press, San Diego.
- Gelvin, S.B. 2000. *Agrobacterium* and Plant Genes Involved in T-DNA Transfer and Integration. *Annu Rev Plant Physiol Plant Mol Biol* 51:223-256.
- Gelvin, S.B. 2003a. *Agrobacterium*-mediated plant transformation: The biology behind the "gene-Jockeying" tool. *Microbiology and Molecular Biology Reviews* 67:16-30.
- Gelvin, S.B. 2003b. Improving plant genetic engineering by manipulating the host. *Trends Biotechnol* 21:95-8.

- Gelvin, S.B. 2005. Agricultural biotechnology: gene exchange by design. *Nature* 433:583-4.
- Gelvin, S.B. 2006. *Agrobacterium* virulence gene induction. *Methods Mol Biol* 343:77-84.
- Gelvin, S.B. 2009. *Agrobacterium* in the genomics age. *Plant Physiol*.
- Gibson, D.M., R.E.B. Ketchum, N.C. Vance, and A.A. Christen. 1993. Initiation and Growth of Cell-Lines of *Taxus brevifolia* (Pacific Yew). *Plant Cell Rep* 12:479-482.
- Gibson, D.M.K., R.E.B. Ketchum, Hirasuna, T.J., and Shuler, M.L. 1995. Taxol: Science and application, p. 71-95, *In* M. Stuffness, ed. *Potential of Plant Cell Culture for Taxane production*, New York.
- Grace, L.J., J.A. Charity, B. Gresham, N. Kay, and C. Walter. 2005. Insect-resistant transgenic *Pinus radiata*. *Plant Cell Rep* 24:103-111.
- Gritz, L., and J. Davies. 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* 25:179-88.
- Hefner, J., S.M. Rubenstein, R.E.B. Ketchum, D.M. Gibson, R.M. Williams, and R. Croteau. 1996. Cytochrome P450-catalyzed hydroxylation of taxa-4(5),11(12)-diene to taxa-4(20),11(12)-dien-5 alpha-ol: The first oxygenation step in taxol biosynthesis. *Chem Biol* 3:479-489.
- Herrera-Estrella, L., J. Simpson, and M. Martinez-Trujillo. 2005. Transgenic plants: an historical perspective. *Methods Mol Biol* 286:3-32.
- Hood, E.E., R.T. Fraley, and M.D. Chilton. 1987. Virulence of *Agrobacterium tumefaciens* strain A281 on legumes. *Journal Name: Plant Physiol* 83:3: 529-534.
- <http://www.cancer.gov>. <http://www.cancer.gov> [Online].
- Humara, J., and R. Ordás. 1999. The toxicity of antibiotics and herbicides on in vitro adventitious shoot formation of *Pinus pinea* L. cotyledons. *In Vitro Cell Dev Biol* 35:339-343.
- Iamtham, S., and A. Day. 2000. Removal of antibiotic resistance genes from transgenic tobacco plastids. *Nat Biotech* 18:1172-1176.
- Ishizaki, K., S. Chiyoda, K.T. Yamato, and T. Kohchi. 2008. *Agrobacterium*-mediated transformation of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. *Plant Cell Physiol* 49:1084-91.

- Jaziri, M., A. Zhiri, Y.W. Guo, J.P. Dupont, K. Shimomura, H. Hamada, M. Vanhaelen, and J. Homes. 1996. *Taxus* sp. cell, tissue and organ cultures as alternative sources for taxoid production: A literature survey. *Plant Cell Tiss Org* 46:59-75.
- Jennewein, S., and R. Croteau. 2001. Taxol: biosynthesis, molecular genetics, and biotechnological applications. *Appl Microbiol Biotechnol* 57:13-19.
- Jennewein, S., C.D. Rithner, R.M. Williams, and R.B. Croteau. 2001. Taxol biosynthesis: Taxane 13 alpha-hydroxylase is a cytochrome P450-dependent monooxygenase. *Proc Natl Acad Sci U S A* 98:13595-13600.
- Jennewein, S., C.D. Rithner, R.M. Williams, and R. Croteau. 2003. Taxoid metabolism: Taxoid 14 beta-hydroxylase is a cytochrome P450-dependent monooxygenase. *Arch Biochem Biophys* 413:262-270.
- Jennewein, S., R.M. Long, R.M. Williams, and R. Croteau. 2004. Cytochrome P450 taxadiene 5 alpha-hydroxylase, a mechanistically unusual monooxygenase catalyzing the first oxygenation step of taxol biosynthesis. *Chem Biol* 11:379-387.
- Jennewein, S., H. Park, J.M. DeJong, R.M. Long, A.P. Bollon, and R.B. Croteau. 2005. Coexpression in yeast of *Taxus* cytochrome p450 reductase with cytochrome P450 oxygenases involved in taxol biosynthesis. *Biotechnol Bioeng* 89:588-598.
- Ji, H.F., X.J. Li, and H.Y. Zhang. 2009. Natural products and drug discovery: Can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? *EMBO Rep* 10:194-200.
- Jones, H.D., and C.A. Sparks. 2009. Stable transformation of plants. *Methods Mol Biol* 513:111-30.
- Kaltenbach, M., G. Schroder, E. Schmelzer, V. Lutz, and J. Schroder. 1999. Flavonoid hydroxylase from *Catharanthus roseus*: cDNA, heterologous expression, enzyme properties and cell-type specific expression in plants. *Plant J* 19:183-93.
- Karimi, M., D. Inze, and A. Depicker. 2002. GATEWAY(TM) vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci* 7:193-195.
- Kaspera, R., and R. Croteau. 2006. Cytochrome P450 oxygenases of Taxol biosynthesis. *Pytochemical Rev* 5:433-444.
- Ketchum, R.E., L. Wherland, and R.B. Croteau. 2007. Stable transformation and long-term maintenance of transgenic *Taxus* cell suspension cultures. *Plant Cell Rep* 26:1025-33.

- Ketchum, R.E., D.M. Gibson, R.B. Croteau, and M.L. Shuler. 1999. The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. *Biotechnol Bioeng* 62:97-105.
- Ketchum, R.E.B., and D.M. Gibson. 1996. Paclitaxel production in suspension cell cultures of *Taxus*. *Plant Cell Tiss Org* 46:9-16.
- Ketchum, R.E.B. and R.B Croteau. 2006. The *Taxus* metabolome and the elucidation off the taxol biosynthetic pathway in cell suspension cultures., p. 291-309, *In* K. D. Saito, R.A.; and Willmitzer, L., ed. *Biotechnology in Agriculture and Forestry*, Vol. 57. Springer-Verlag, Berlin.
- Khosroushahi, A.Y., M. Valizadeh, A. Ghasempour, M. Khosrowshahli, H. Naghdibadi, M.R. Dadpour, and Y. Omid. 2006. Improved taxol production by combination of inducing factors in suspension cell culture of *Taxus baccata*. *Cell Biol Int* 30:262-269.
- Kim, B.J., D.M. Gibson, and M.L. Shuler. 2004. Effect of subculture and elicitation on instability of taxol production in *Taxus* sp. suspension cultures. *Biotechnol Prog* 20:1666-73.
- Kim, B.J., D.M. Gibson, and M.L. Shuler. 2005. Relationship of viability and apoptosis to taxol production in *Taxus* sp. suspension cultures elicited with methyl jasmonate. *Biotechnol Prog* 21:700-7.
- Kim, B.J., D.M. Gibson, and Schuler, M.L. 2004. Effect of subculture and elicitation on instability of taxol production in *Taxus* spp. suspension culture. *Biotechnol Prog* 20:1666-1673.
- Kim, C.H., K.I. Kim, and I.S. Chung. 2000. Expression of modified green fluorescent protein in suspension culture of *Taxus cuspidata*. *J microbiol Biotechnol* 10:91-94.
- Klein, T.M., R. Arentzen, P.A. Lewis, and S. Fitzpatrickmcelligott. 1992. Transformation of microbes, plants and animals by particle bombardment. *Bio-Technology* 10:286-291.
- Kolewe, M., M. Henson, and S. Roberts. 2010. Characterization of aggregate size in *Taxus* suspension cell culture. *Plant Cell Rep* 29:485-494.
- Kolewe, M.E., V. Gaurav, and S.C. Roberts. 2008. Pharmaceutically active natural product synthesis and supply via plant cell culture technology. *Mol Pharm* 5:243-56.

- Kovacs, K., L. Zhang, R.S. Linforth, B. Whittaker, C.J. Hayes, and R.G. Fray. 2007. Redirection of carotenoid metabolism for the efficient production of taxadiene [taxa-4(5),11(12)-diene] in transgenic tomato fruit. *Transgenic Res* 16:121-6.
- Krolikowski, K.A., J.L. Victor, T.N. Wagler, S.J. Lolle, and R.E. Pruitt. 2003. Isolation and characterization of the *Arabidopsis* organ fusion gene HOTHEAD. *Plant J* 35:501-511.
- Lacroix, B., J. Li, T. Tzfira, and V. Citovsky. 2006. Will you let me use your nucleus? How *Agrobacterium* gets its T-DNA expressed in the host plant cell. *J Physiol Pharmacol* 84:333-45.
- Lambardi, M., D. Lachance, A. Seguin, and P.J. Charest. 1998. Evaluation of microprojectile-mediated DNA delivery and reporter genes for genetic transformation of the Mediterranean cypress (*Cupressus sempervirens* L.). *Plant Cell Rep* 18:198-202.
- Larkin, P., and W. Scowcroft. 1981. Somaclonal variation — a novel source of variability from cell cultures for plant improvement. *Theor Appl Genet* 60:197-214.
- LeFrock, J.P., RA; and Leff, RD. 1983. Mechanism of action, antimicrobial activity, pharmacology, adverse effects, and clinical efficacy of cefotaxime. *Pharmacotherapy* 2:174-184.
- Leonard, E., Y. Yan, and M.A.G. Koffas. 2006. Functional expression of a P450 flavonoid hydroxylase for the biosynthesis of plant-specific hydroxylated flavonols in *Escherichia coli*. *Metab Eng* 8:172-181.
- Lichtenthaler, H.K. 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annu Rev Plant Physiol Plant Mol Biol* 50:47-65.
- Lin, X.Y., M. Hezari, A.E. Koepp, H.G. Floss, and R. Croteau. 1996. Mechanism of taxadiene synthase, a diterpene cyclase that catalyzes the first step of taxol biosynthesis in Pacific yew. *Biochemistry* 35:2968-2977.
- Linden, J.C., and M. Phisalaphong. 2000. Oligosaccharides potentiate methyl jasmonate-induced production of paclitaxel in *Taxus canadensis*. *Plant Sci* 158:41-51.
- Ling, H.Q., D. Kriseleit, and M.W. Ganai. 1998. Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* Mill.). *Plant Cell Rep* 17:843-847.
- Liu, P., and E.W. Nester. 2006. Indoleacetic acid, a product of transferred DNA, inhibits vir gene expression and growth of *Agrobacterium tumefaciens* C58. *Proc Natl Acad Sci U S A* 103:4658-62.

- Loncaric, C., E. Merriweather, and K.D. Walker. 2006. Profiling a taxol pathway 10 beta-acetyltransferase: Assessment of the specificity and the production of baccatin III by in vivo acetylation in *E. coli*. *Chem Biol* 13:309-317.
- Long, R.M., and R. Croteau. 2005. Preliminary assessment of the C13-side chain 2'-hydroxylase involved in Taxol biosynthesis. *Biochem Biophys Res Commun* 338:410-417.
- Long, R.M., C. Lagisetti, R.M. Coates, and R.B. Croteau. 2008. Specificity of the N-benzoyl transferase responsible for the last step of Taxol biosynthesis. *Arch Biochem Biophys* 477:384-9.
- Lorence, A., and R. Verpoorte. 2004. Gene transfer and expression in plants. *Methods Mol Biol* 267:329-50.
- Lowe, B., N. Shiva Prakash, M. Way, M. Mann, T. Spencer, and R. Boddupalli. 2009. Enhanced single copy integration events in corn via particle bombardment using low quantities of DNA. *Transgenic Res* 18:831-840.
- Lu, G., Q. Zou, D. Guo, X. Zhuang, X. Yu, X. Xiang, and J. Cao. 2007. *Agrobacterium tumefaciens*-mediated transformation of *Narcissus tazetta* var. *chinensis*. *Plant Cell Rep* 26:1585-93.
- Luehrsen, K.a.W., V. 1992. Firefly luciferase as a reporter for plant gene studies. *Promega Notes Magazine* 44:24-32.
- Luo, J., and G.Y. He. 2004. Optimization of elicitors and precursors for paclitaxel production in cell suspension culture of *Taxus chinensis* in the presence of nutrient feeding. *Process Biochem* 39:1073-1079.
- Malonga, H., J.F. Neault, S. Diamantoglou, and H.A. Tajmir-Riahi. 2005. Taxol anticancer activity and DNA binding. *Mini-Rev Med Chem* 5:307-311.
- Mardamshin, A.G., N.A. Urazbakhtina, and M.S. Yumaguzhin. 2001. Polynucleate cells in the callus tissue of *Taxus baccata* L. *Dokl Biol Sci* 381:576-8.
- Mathias, R.J., and L.A. Boyd. 1986. Cefotaxime stimulates callus growth, embryogenesis and regeneration in hexaploid bread wheat (*Triticum aestivum* L em. thell). *Plant Sci* 46:217-223.
- Mathias, R.J., and C. Mukasa. 1987. The effect of cefotaxime on the growth and regeneration of callus from four varieties of barley (*Hordeum vulgare* L.). *Plant Cell Rep* 6:454-457.

- McGaha, S.M., and W.S. Champney. 2007. Hygromycin B inhibition of protein synthesis and ribosome biogenesis in *Escherichia coli*. *Antimicrob Agents Chemother* 51:591-6.
- Menhard, B.Z., M.H. 1998. Purification and characterization of acetyl coenzyme A: 10-hydroxytaxane O-acetyltransferase from cell suspension cultures of *Taxus chinensis*. *Phytochemistry* 50:763-774.
- Miller, K., B. Neilan, and D.M. Sze. 2008. Development of Taxol and other endophyte produced anti-cancer agents. *Recent Pat Anticancer Drug Discov* 3:14-9.
- Montague, A., A. Ziauddin, R. Lee, W. Ainley, and J. Strommer. 2007. High-efficiency phosphinothricin-based selection for alfalfa transformation. *Plant Cell Tiss Org* 91:29-36.
- Naill, M.C., and S.C. Roberts. 2005. Flow cytometric identification of paclitaxel-accumulating subpopulations. *Biotechnol Prog* 21:978-983.
- Navia-Orsorio, A., H. Garden, R.M. Cusido, J. Palazon, A.W. Alfermann, and M.T. Pinol. 2002. Production of paclitaxel and baccatin III in a 20-L airlift bioreactor by a cell suspension of *Taxus wallichiana*. *Planta Med* 68:336-40.
- Nawarskas, J.J., and L.A. Osborn. 2005. Paclitaxel-eluting stents in coronary artery disease. *Am J Health-Syst Ph* 62:2241-2251.
- Nehra, N.S., M.R. Becwar, W.H. Rottmann, L. Pearson, K. Chowdhury, S.J. Chang, H.D. Wilde, R.J. Kodrzycki, C.S. Zhang, K.C. Gause, D.W. Parks, and M.A. Hinchee. 2005. Forest biotechnology: Innovative methods, emerging opportunities. *In Vitro Cell Dev Biol-Plant* 41:701-717.
- Nelson, B.K., X. Cai, and A. Nebenfuhr. 2007. A multicolored set of in vivo organelle markers for co-localization studies in *Arabidopsis* and other plants. *Plant J* 51:1126-36.
- Nigro, S.A., N.P. Makunga, N.B. Jones, and J. van Staden. 2004. A biolistic approach towards producing transgenic *Pinus patula* embryonal suspensor masses. *Plant Growth Regul* 44:187-197.
- Nigro, S.A., N.P. Makunga, N.B. Jones, and J.V. Staden. 2008. An *Agrobacterium*-mediated system for gene transfer in *Pinus patula*. *S Afr J Bot* 74:144-148.
- Nims, E., C.P. Dubois, S.C. Roberts, and E.L. Walker. 2006. Expression profiling of genes involved in paclitaxel biosynthesis for targeted metabolic engineering. *Metab Eng* 8:385-94.

- Nims, E., C.P. Dubois, S.C. Roberts, and E.L. Walker. 2007. Expression profiling of genes involved in paclitaxel biosynthesis for targeted metabolic engineering. *Metab Eng* 8:385-94.
- Oberlies, N.H., and D.J. Kroll. 2004. Camptothecin and taxol: historic achievements in natural products research. *J Nat Prod* 67:129-35.
- Ortholand, J.-Y., and A. Ganesan. 2004. Natural products and combinatorial chemistry: back to the future. *Curr Opin Chem Biol* 8:271-280.
- Oshige, M., M. Takenouchi, Y. Kato, S. Kamisuki, T. Takeuchi, K. Kuramochi, I. Shiina, Y. Suenaga, Y. Kawakita, K. Kuroda, N. Sato, S. Kobayashi, F. Sugawara, and K. Sakaguchi. 2004. Taxol derivatives are selective inhibitors of DNA polymerase alpha. *Bioorg Med Chem* 12:2597-2601.
- Palazon, J., R.M. Cusido, M. Bonfill, C. Morales, and M.T. Pinol. 2003. Inhibition of paclitaxel and baccatin III accumulation by mevinolin and fosmidomycin in suspension cultures of *Taxus baccata*. *J Biotechnol* 101:157-63.
- Pornprom, T., N. Prodmatee, and O. Chatchawankanphanich. 2009. Glutamine synthetase mutation conferring target-site-based resistance to glufosinate in soybean cell selections. *Pest Manage Sci* 65:216-222.
- Pyo, S.K., M.; Cho, J.; Song, B.; Han, B.; and Choi, H. 2004. *J Chem Technol Biotechnol* 79:1162-1168.
- Qian, Z.G., Z.J. Zhao, Y. Xu, X. Qian, and J.J. Zhong. 2004. Novel chemically synthesized hydroxyl-containing jasmonates as powerful inducing signals for plant secondary metabolism. *Biotechnol Bioeng* 86:809-16.
- Qian, Z.G., Z.J. Zhao, Y. Xu, X. Qian, and J.J. Zhong. 2005. A novel synthetic fluoro-containing jasmonate derivative acts as a chemical inducing signal for plant secondary metabolism. *Appl Microbiol Biotechnol* 68:98-103.
- Qin, W.M., and W.Z. Lan. 2004. Fungal elicitor-induced cell death in *Taxus chinensis* suspension cells is mediated by ethylene and polyamines. *Plant Sci* 166:989-995.
- Radke, S.E., J.C. Turner, and D. Facciotti. 1992. Transformation and regeneration of *Brassica rapa* Using *Agrobacterium tumefaciens*. *Plant Cell Rep* 11:499-505.
- Rajam, M.V., and S.V. Kumar. 2006. Green Alga (*Chlamydomonas reinhardtii*). *Methods Mol Biol* 344:421-33.
- Rakoczy-Trojanowska, M. 2002. Alternative methods of plant transformation - A short review. *Cell Mol Biol Lett* 7:849-858.

- Rasco-Gaunt, S., A. Riley, P. Barcelo, and P.A. Lazzeri. 1999. Analysis of particle bombardment parameters to optimize DNA delivery into wheat tissues. *Plant Cell Rep* 19:118-127.
- Ro, D.-K., E.M. Paradise, M. Ouellet, K.J. Fisher, K.L. Newman, J.M. Ndungu, K.A. Ho, R.A. Eachus, T.S. Ham, J. Kirby, M.C.Y. Chang, S.T. Withers, Y. Shiba, R. Sarpong, and J.D. Keasling. 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440:940-943.
- Roberts, S.C. 2007. Production and engineering of terpenoids in plant cell culture. *Nat Chem Biol* 3:387-95.
- Roberts, S.C., and M.L. Shuler. 1997. Large-scale plant cell culture. *Curr Opin Biotechnol* 8:154-9.
- Roberts, S.C., M. Naill, D.M. Gibson, and M.L. Shuler. 2003. A simple method for enhancing paclitaxel release from *Taxus canadensis* cell suspension cultures utilizing cell wall digesting enzymes. *Plant Cell Rep* 21:1217-1220.
- Rontein, D., S. Onillon, G. Herbette, A. Lesot, D. Werck-Reichhart, C. Sallaud, and A. Tissier. 2008. CYP725A4 from Yew Catalyzes Complex Structural Rearrangement of Taxa-4(5),11(12)-diene into the Cyclic Ether 5(12)-Oxa-3(11)-cyclotaxane. *J Biol Chem* 283:6067-75.
- Salaj, T., J. Moravcikova, L. Grec-Niquet, and J. Salaj. 2005. Stable transformation of embryogenic tissues of *Pinus nigra* Arn. using a biolistic method. *Biotechnol Lett* 27:899-903.
- Sawers, R.J., P.R. Farmer, P. Moffett, and T.P. Brutnell. 2006. In planta transient expression as a system for genetic and biochemical analyses of chlorophyll biosynthesis. *Plant Methods* 2:15.
- Schoendorf, A., C.D. Rithner, R.M. Williams, and R.B. Croteau. 2001. Molecular cloning of a cytochrome P450 taxane 10 beta-hydroxylase cDNA from *Taxus* and functional expression in yeast. *Proc Natl Acad Sci U S A* 98:1501-1506.
- Sheludko, Y.V. 2008. *Agrobacterium*-mediated transient expression as an approach to production of recombinant proteins in plants. *Recent Pat Biotechnol* 2:198-208.
- Stadler, R., and M.H. Stenk. 1993. The purification and characterization of a unique cytochrome P-450 enzyme from *Berberis stolonifera* plant cell cultures. *J Biol Chem* 268:823-831.

- Steele, C.L., Y.J. Chen, B.A. Dougherty, W.Y. Li, S. Hofstead, K.S. Lam, Z.H. Xing, and S.J. Chiang. 2005. Purification, cloning, and functional expression of phenylalanine aminomutase: The first committed step in Taxol side-chain biosynthesis. *Arch Biochem Biophys* 438:1-10.
- Sundar, I.K., and N. Sakthivel. 2008a. Advances in selectable marker genes for plant transformation. *J Plant Physiol* 165:1698-1716.
- Tabata, H. 2004. Paclitaxel production by plant-cell-culture technology. *Adv Biochem Eng Biotechnol* 87:1-23.
- Tabata, H. 2006. Production of paclitaxel and the related taxanes by cell suspension cultures of *Taxus* species. *Curr Drug Targets* 7:453-61.
- Tao, L.Z., A.Y. Cheung, and H.M. Wu. 2002. Plant Rac-like GTPases are activated by auxin and mediate auxin-responsive gene expression. *Plant Cell* 14:2745-60.
- Tee, C.S., and M. Maziah. 2005. Optimization of biolistic bombardment parameters for *Dendrobium Sonia* 17 calluses using GFP and GUS as the reporter system. *Plant Cell Tiss Org* 80:77-89.
- Thompson, C.J., N.R. Movva, R. Tizard, R. Cramer, J.E. Davies, M. Lauwereys, and J. Botterman. 1987. Characterization of the Herbicide-Resistance Gene Bar from *Streptomyces hygroscopicus*. *EMBO J* 6:2519-2523.
- Tzfira, T., and V. Citovsky. 2006. *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Curr Opin Biotechnol* 17:147-54.
- van der Fits, L., and J. Memelink. 2000. ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science* 289:295-297.
- van der Fits, L., E.A. Deakin, J.H.C. Hoge, and J. Memelink. 2000. The ternary transformation system: constitutive virG on a compatible plasmid dramatically increases *Agrobacterium*-mediated plant transformation. *Plant Mol Biol* 43:495-502.
- van Rozendaal, E.L., G.P. Lelyveld, and T.A. van Beek. 2000. Screening of the needles of different yew species and cultivars for paclitaxel and related taxoids. *Phytochemistry* 53:383-9.
- Vogel, J., and T. Hill. 2008. High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3. *Plant Cell Rep* 27:471-478.
- Vongpaseuth, K., and S.C. Roberts. 2007. Advancements in the understanding of Paclitaxel metabolism in tissue culture. *Curr Pharm Biotechnol* 8:219-36.

- Vongpaseuth, K., E. Nims, M. St Amand, E.L. Walker, and S.C. Roberts. 2007. Development of a particle bombardment-mediated transient transformation system for *Taxus* spp. cells in culture. *Biotechnol Prog* 23:1180-5.
- Walker, K., and R. Croteau. 2000a. Molecular cloning of a 10-deacetylbaccatin III-10-O-acetyl transferase cDNA from *Taxus* and functional expression in *Escherichia coli*. *Proc Natl Acad Sci U S A* 97:583-587.
- Walker, K., and R. Croteau. 2000b. Taxol biosynthesis: Molecular cloning of a benzoyl-CoA : taxane 2 alpha-O-benzoyltransferase cDNA from *Taxus* and functional expression in *Escherichia coli*. *Proc Natl Acad Sci U S A* 97:13591-13596.
- Walker, K., A. Schoendorf, and R. Croteau. 2000. Molecular cloning of a taxa-4(20),11(12)-dien-5 alpha-ol-O-acetyl transferase cDNA from *Taxus* and functional expression in *Escherichia coli*. *Arch Biochem Biophys* 374:371-380.
- Walker, K., R. Long, and R. Croteau. 2002a. The final acylation step in Taxol biosynthesis: Cloning of the taxoid C13-side-chain N-benzoyltransferase from *Taxus*. *Proc Natl Acad Sci U S A* 99:9166-9171.
- Walker, K., S. Fujisaki, R. Long, and R. Croteau. 2002b. Molecular cloning and heterologous expression of the C-13 phenylpropanoid side chain-CoA acyltransferase that functions in Taxol biosynthesis. *Proc Natl Acad Sci U S A* 99:12715-12720.
- Walker, K., R.E.B. Ketchum, M. Hezari, D. Gatfield, M. Goleniowski, A. Barthol, and R. Croteau. 1999. Partial purification and characterization of acetyl coenzyme A: Taxa-4(20),11(12)-dien-5 alpha-ol O-acetyl transferase that catalyzes the first acylation step of Taxol biosynthesis. *Arch Biochem Biophys* 364:273-279.
- Walker, K.D., K. Klettke, T. Akiyama, and R. Croteau. 2004. Cloning, heterologous expression, and characterization of a phenylalanine aminomutase involved in Taxol biosynthesis. *J Biol Chem* 279:53947-54.
- Wang, C.W., J.; and Mei, X. 2001. *Appl Microbiol Biotechnol* 55:404-410.
- Wang, Y.D., J.C. Wu, and Y.J. Yuan. 2007. Salicylic acid-induced taxol production and isopentenyl pyrophosphate biosynthesis in suspension cultures of *Taxus chinensis* var. mairei. *Cell Biol Int* 31:1179-83.
- Wenck, A.R., M. Quinn, R.W. Whetten, G. Pullman, and R. Sederoff. 1999. High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). *Plant Mol Biol* 39:407-16.
- Widholm, J.M. 1972. Use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology* 47:189-&.

- Wildung, M.R., and R. Croteau. 1996. A cDNA clone for taxadiene synthase, the diterpene cyclase that catalyzes the committed step of taxol biosynthesis. *J Biol Chem* 271:9201-9204.
- Wood, C.C., J.R. Petrie, P. Shrestha, M.P. Mansour, P.D. Nichols, A.G. Green, and S.P. Singh. 2009. A leaf-based assay using interchangeable design principles to rapidly assemble multistep recombinant pathways. *Plant Biotechnol J* 7:914-24.
- Wu, J., C. Wang, and X. Mei. 2001. Stimulation of taxol production and excretion in *Taxus* spp cell cultures by rare earth chemical lanthanum. *J Biotechnol* 85:67-73.
- Wydro, M., E. Kozubek, and P. Lehmann. 2006. Optimization of transient *Agrobacterium*-mediated gene expression system in leaves of *Nicotiana benthamiana*. *Acta Biochim Pol* 53:289-98.
- Xiang, F., L.J. Yu, W. Chen, and Z. Liu. 2008. Effect of cell culture on 18S rRNA gene sequences in the cultural course of *Taxus chinensis* cells. *Z Naturforsch [C]* 63:127-32.
- Yajun, Y.A.N., Chemler, Joseph, H. Lixuan, Martens, Stefan, Koffas, and A.G. Mattheos. 2005. Metabolic engineering of anthocyanin biosynthesis in *Escherichia coli* American Society for Microbiology, Washington, DC, ETATS-UNIS.
- Yang, F.J., H.H. Pang, X.K. Zhang, J.Y. Sun, and Y.G. Zu. 2008. Quantitative changes of anti-cancer active components in *Taxus chinensis* var. mairei branches and leaves. *Ying Yong Sheng Tai Xue Bao* 19:911-4.
- Yang, Y.N., R.G. Li, and M. Qi. 2000. In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. *Plant J* 22:543-551.
- Yin, X., and Z.J. Zhang. 2010. Recent Patents on Plant Transgenic Technology. *Recent Patents on Biotechnology* 4:98-111.
- Yuan, Y.W., Z.; Miao, Z.; and Wu, J. 2002. *Biochem Eng J* 10:77-83.
- Yuan, Z.C., M.P. Edlind, P. Liu, P. Saenkham, L.M. Banta, A.A. Wise, E. Ronzone, A.N. Binns, K. Kerr, and E.W. Nester. 2007. The plant signal salicylic acid shuts down expression of the vir regulon and activates quorum-quenching genes in *Agrobacterium*. *Proc Natl Acad Sci U S A* 104:11790-5.
- Yukimune, Y., H. Tabata, Y. Higashi, and Y. Hara. 1996. Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. *Nat Biotechnol* 14:1129-1132.

- Zhang, C.H., X.G. Mei, L. Liu, and L.J. Yu. 2000. Enhanced paclitaxel production induced by the combination of elicitors in cell suspension cultures of *Taxus chinensis*. *Biotechnol Lett* 22:1561-1564.
- Zhang, P., P.P. Zhou, C. Jiang, H. Yu, and L.J. Yu. 2008. Screening of Taxol-producing fungi based on PCR amplification from *Taxus*. *Biotechnol Lett* 30:2119-23.
- Zhao, C.F., L.J. Yu, Z. Liu, and Y.P. Sun. 2006. The dynamic variation of several important taxane content in post-harvest *Taxus chinensis* clippings. *J Asian Nat Prod Res* 8:229-39.
- Zhong, J.J. 2002. Plant cell culture for production of paclitaxel and other taxanes. *J Biosci Bioeng* 94:591-9.